Development of the first vertebrate epithelium

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Summary

A conserved feature of early vertebrate embryos is the formation of simple epithelial layer of cells which surrounds the embryo and protects it from the external environment. This epithelium is called the trophectoderm in mammals, the superficial layer in *Xenopus* and the enveloping layer in zebrafish. This project investigates what promotes differentiation of this cell type. In *Xenopus* embryos aPKC and Notch signalling were found to be unable to promote differentiation of the superficial layer. In contrast, BMP signalling can promote expression of a number of transcriptional regulators, including members of the Grhl and Msx families and differentiation of the superficial layer. This pathway is initiated in the underlying deep cells, but not all target genes are activated so differentiation does not occur in these cells. The role of BMP signalling in mouse development was investigated by using mouse embryonic stem cells (mESCs) as the model. BMP4 is sufficient to induce mESCs to form a polarised epithelial cell type and that these epithelial cells appear trophoblast in fate. BMP signalling activates *Grhl* and *Msx* genes in mESCs, as it does in *Xenopus* embryos. This suggests that similar target genes are activated by BMP signalling in the first epithelium of *Xenopus* and mouse. Based on this data it is tempting to propose that BMP signalling acts in a conserved manor to promote differentiation of the first epithelium in diverse vertebrates.

Abbreviation

ALK2: Activin receptor - Like Kinase-2

ALK3: Activin receptor - Like Kinase-3

ALK6: Activin receptor - Like Kinase-6

AP: Anterior-Posterior

aPKC: atypical Protein Kinase C

BMPs: Bone Morphogenetic Proteins

Co-Smad: Common-partner Smad4

CRB: Crumbs

CtBP: C-terminal Binding Protein

DEL: Deep Layers

DLG: Discs large

Dlx: Distal-less

DSL: Delta，Serrate/Jagged，Lag-2

DV: Dorso-Ventral

EMT: Epithelial-Mesenchymal Transition

ERK: Extracellular signal-Regulated protein Kinase

ESCRT: Endosomal Sorting Complex Required for Transport

ESCs: Embryonic Stem Cells

EVL: Enveloping Layer

FGF: Fibroblast Growth Factor

FRS2α: Fibroblast Growth Factor Receptor substrate 2α

GMC: Ganglion Mother Cell

Grhl1: Grainyhead-like 1

Grhl3: Grainyhead-like 3

hESCs: human Embryonic Stem Cells

HSCs: Hematopoietic Stem Cells

Hugl-1: Human Lgl1

I-Smads: Inhibitory Smads

ICM: Inner Cell Mass

Id: Inhibitor of Differentiation family

Irf6: Interferon Regulatory Factor 6

JAMS: Junctional Adhesion Molecules

LEF1: Lymphoid Enhancer binding Factor 1

LGL: Lethal Giant Larvae

LIF: Leukemia Inhibitory Factor

LMZ: Lateral Marginal Zones

LR: Left-Right

MAPK: Mitogen-Activated Protein Kinase

MAPKKK: Mitogen-Activated Protein Kinase Kinase Kinase

MDCK: Madin Darby Canine Kidney

mEpiSCs: mouse Epiblast Stem Cells

mESCs: mouse Embryonic Stem Cells

MOs: Morpholino Oligonucleotides

Msh: Muscle Segment Homeobox

Notch-ICD: Notch Inter-Cellular Domain

Oct3/4: Octamer-4

ODC: Ornithine decarboxylase

OvCa: Ovarian Cancer

PAR: Partitioning-defective

PI3k: Phosphatidylinosital-3 kinase

PL-2: Placental Lactogen-2

Pou5f1: POU domain class 5 transcription factor 1

PSE: Primary Superficial Epithelium

PTEN: Phosphatase and Tensin homolog

R-Smads: Receptor-regulated Smads

SCRIB: Scribble

Sip1: Smad-Interacting Protein-1

SOP: Sensory Organ Precursor

Sox: SRY-related HMG box

STAT3: Signal Transducer and Activator of Transcription-3

TAK1: TGF-β-Activated Kinase 1

TE: trophectoderm

TGF-β: Transforming Growth Factor β

TJ: tight junction

VMZ: Ventral Marginal Zones

X-TSK: X-Tsukushi

Yap1: Yes-Associated Protein 1

ZA: Zonula adherens

ZO: Zona Occludens

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1. Chapter I Introduction

The first differentiative event to occur in early vertebrate embryos is the formation of a simple epithelial layer which surrounds the embryo and protects it from the external environment (Muller, 2001). This project will investigate the factors which promote differentiation of this epithelium. The introduction will start by reviewing a number of areas that are important for understanding the formation of the first epithelium, before focusing specifically on this epithelium in *Xenopus*, Zebrafish and mouse.

1.1 Epithelial cell structure

Epithelial cells are in many organs of the vertebrate, from the skin to the blood vessels. Epithelial cells are important for providing barriers, between different regions, or the outer layer of an organism and the external environment.

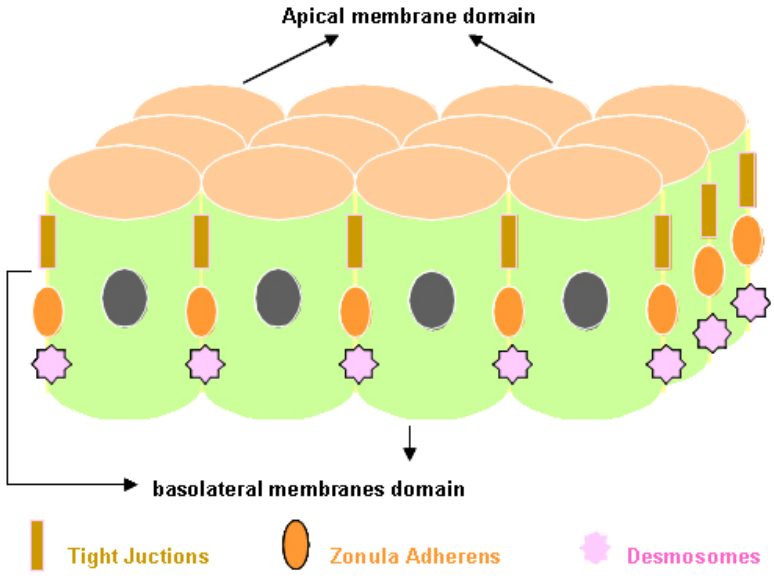


Fig 1 Epithelial cells structure. Epithelial cell polarity is defined by distinct apical and basolateral domains which separate by an adhesive belt. The adhesive belt consists of Tight Junction (TJ), Zonula adherens (ZAs) and desmosomes (reviewed by Knust and Bossinger, 2002)

Key structures are necessary for epithelial cells to provide a barrier (Fig. 1). For

example, epithelial cells exhibit cell polarity, which is defined by distinct apical and basolateral domains. An adhesive belt encircling the cell lies below the apical surface. The belt consists of Tight Junction (TJ), Zonula adherens (ZAs) and desmosomes (reviewed by Knust and Bossinger, 2002; shown in Fig 1).

ZAs, also called belt-like adherens junctions, connect the actin cytoskeleton of neighboring cells (reviewed by Zhang et al., 2005). ZAs are composed of E-cadherin and catenins. E-cadherin is a transmembrane protein, forming homodimers with other cadherin molecules on adjacent cells (Leckband and Sivasankar, 2000). The intracellular domain of E-cadherin directly binds to the actin cytoskeleton (reviewed by Leckband and Sivasankar, 2000). Two catenins and an adaptor protein plakoglobin are essential for maintaining ZAs function. β-catenin (or plakoglobin) interacts with the cadherin via catenin-binding region. α-catenin binds actin and the cadherin indirectly via β-catenin (Yamada et al., 2005).

TJs localise to the apical side of ZAs, and establish an apical-basolateral barrier to diffusion across the epithelial sheet. TJs not only restrict the movement of membrane molecules between the apical and basolateral domain (reviewed by Köhler and Zahraoui, 2005), but also play an organizing role in epithelial polarization (reviewed by Köhler and Zahraoui, 2005). Four types of transmembrane proteins are found in TJs, including Occludin, Claudins, JAMs (Junctional Adhesion Molecules) and Crb (Crumbs) (reviewed by Köhler and Zahraoui, 2005). Other than transmembrane proteins, TJs contain various types of membrane associated proteins, including members of the PDZ domain containing protein ZO (Zona Occludens) family. Those proteins link transmembrane proteins with the underlying cytoskeleton (Wittchen et

al., 1999), or recruit regulatory proteins to regulate cell behaviour. Thus, TJs are no longer regarded as a simple barrier and it is largely accepted that they constitute multifunctional complexes involved in various signalling events controlling cell–cell adhesion, differentiation and polarity (reviewed by Köhler and Zahraoui, 2005).

Other than TJs and ZAs, the desmosome is another component of the "barrier" structure between cells, which is underneath the other two. The desmosome is also composed of adhesion proteins, such as desmosomal cadherins, cytoplasm proteins, and plakin family of cytolinkers (reviewed by Chidgey and Dawson, 2007). The cytoplasmic proteins of desmosomes, including desmoplakin, plakoglobin and plakophilins, interact with desmosomal cadherins and the C-terminal domain of desmoplakin interacts with intermediate filaments (reviewed by Chidgey and Dawson, 2007).

1.2 Asymmetric cell division and cell polarity

An important question in understanding the development of epithelial tissues is how the balance between proliferation and differentiation is regulated and how cells of different lineages are produced. One mechanism of regulating these processes is asymmetric cell division. During this process one mother cell produces two daughter cells with different cell fates by asymmetrically segregating fate determinants into different daughter cells. A well studied example occurs in early *C. elegans* development, the three principal axes of the body plan are established by a series of asymmetric divisions, which include the axes of the anterior-posterior (AP), the dorso-ventral (DV) and the left-right (LR) (reviewed by Betschinger and Knoblich,

2004).

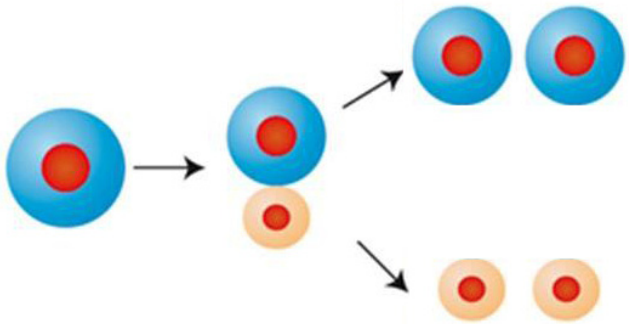


Fig 2 A model for asymmetric cell division. A mother cell divides to produce two daughter cells, they differ in fate. One daughter maintains the same fate as the mother (blue and big), while the other daughter cell follows another fate (pink and small).

There are three basic requirements for this mechanism: an axis of polarity is established within the cell, the mitotic spindle aligns with this axis of polarity, and cell fate determinants become distributed asymmetrically along the axis of polarity so that they are differentially inherited by the daughter cells (Betschinger and Knoblich, 2004). An alternative form of asymmetric cell division also occurs, where extrinsic cues are involved. In the stem cell niches of *Drosophila* testes and ovaries, extrinsic cues come from inductive signals produced by surrounding tissues, (Yamashita et al., 2003; Chen and McKearin, 2003).

1.2.1 Cell polarity

Establishment of cell polarity is the first and fundamental step for a cell to undergo asymmetric cell division and is also a defining feature of epithelial cells (see section 1.1). Polarization makes it possible for cell fate determinants to be asymmetrically segregated into one daughter cell. Cell polarity is also involved in biological events as diverse as chemotaxis of bacteria, nutrient absorption, axon guidance and oogenesis

(reviewed by Pamela and Suzanne, 2004).

Although found in diverse cell types and species, cell polarization appears to involve conserved regulators. A core regulator of cell polarization is the PAR-aPKC complex. The PAR-aPKC complex comprises of the serine/threonine protein kinase aPKC and two scaffold proteins PAR-3 and PAR-6 (reviewed by Suzuki and Ohno, 2006). They interact with each other to form an active working unit (reviewed by Suzuki and Ohno, 2006). aPKC interacts with PAR-6 through its N-terminal PB1 domains (Suzuki et al., 2003), but interacts with PAR-3 through its central conserved region which contains the kinase domain (Izumi et al., 1998). An additional interaction also happens between the PDZ domains of PAR-3 and PAR-6 (Lin et al., 2000). On the other hand, PAR-3 has also been found at distinct locations from aPKC and PAR-6 in polarized cells, showing that aPKC and PAR6 do not always bind PAR3 (Harris and Peifer, 2005). It appears that PAR6 recruits specific substrates for aPKC, including Par3 and other targets, and then aPKC phosphorylates the target protein.

In *Drosophila* epithelial cells, the PAR-aPKC complex localises in the apical membrane domain (Petronczki and Knoblich, 2001). A similar localization of the PAR-aPKC complex is also found in neuroblasts (Albertson and Doe, 2003). Rolles and colleague revealed that *Drosophila* aPKC zygotic null mutants have polarity defects in both neuroblasts and imaginal disc epithelial cells (Rolles et al., 2003). This data demonstrates that aPKC has a key role in regulating cell polarity in *Drosophila*.

The PAR-aPKC complex cooperates with other conserved proteins to regulate polarity. In *Drosophila*, PAR-1 phosphorylates PAR-3 on its conserved serine residues and

induces the aPKC complex destabilization (Benton and St Johnston, 2003). This antagonistic role restricts aPKC localization to the apical complex (Cuenca et al., 2003). Lethal giant larvae (Lgl), Scribble (Scrib) and Discs large (Dlg) are tumor suppressor proteins in *Drosophila* (Bilder and Perrimon, 2000). They localise in the basolateral membrane domain of epithelial cells (Bilder and Perrimon, 2000). In both larval and embryonic *Drosophila* neuroblasts, Dlg, Scrib, and Lgl proteins display a uniform cortical localization (Albertson and Doe, 2003). Lgl is one target of PAR-aPKC complex (Betschinger et al., 2003). Phosphorylation by PAR-aPKC inactivates the Lgl and causes it to be released from membranes in epithelial cells. On the other hand, Lgl can inactivate aPKC by forming Lgl–PAR6–aPKC complex (Betschinger et al., 2003). The antagonism also occurs in neuroblasts, but the mechanism does not change their localisation (Peng et al., 2002; Lee et al., 2006). Thus, aPKC and Lgl act antagonistically to establish polarity and allow fate determinant segregation: Lgl is inactivated in the apical cell cortex where PAR-aPKC localises; however, Lgl is still unphosphorylated and active in the basal domain to maintain aPKC apical localization. This suggests that mutual inhibition between apical and basal lateral proteins acts to maintain distinct membrane domains.

Recent progress has shown that the PAR-aPKC system displays similar functions in mammalian cells as well as in *Drosophila*. In mammalian epithelial cells, coupling the aPKC complex to junctional structures is essential for epithelial polarity (Ebnet et al., 2004; Itoh et al., 2001). In MDCK cells (Madin Darby Canine Kidney cells), mammalian aPKC acts up-stream of PAR-1b (one of the PAR-1 variants) to establish and maintain the epithelial cell polarity (Suzuki et al., 2004). The interaction between aPKC and LGL was also demonstrated to be essential to establish mammalian

epithelial polarity, LGL inhibits the apical PAR-3-aPKC-PAR-6 complex activity while aPKC maintains LGL in the lateral region to form the basolateral membrane identity (Yamanaka et al., 2006).

1.2.2 Spindle orientation

The asymmetric distribution of cell fate determinants is not sufficient for asymmetric division, the mitotic spindle needs to align along the polarity axis (Ahringer, 2003). Research in different organisms has revealed that cooperation between the aPKC complex and heterotrimeric G proteins play a key role in adjusting the orientation of the mitotic spindle (Grill et al., 2003). One model is that the PAR-aPKC complex and heterotrimeric G proteins regulate the cell cortex and microtubules interaction to adjust spindle position (Schaefer et al, 2000; Grill et al., 2003).

1.2.3 Cell fate determinants segregation

The key point of an asymmetric cell division is the segregation of cell fate determinant molecules into one of the two daughter cells. For example, in *Drosophila* neuroblast, one asymmetric cell division produces a self-renewing neuroblast and a differentiated ganglion mother cell (GMC). *aPKC*, in addition to regulating polarity, acts as a neuroblast fate determinant (Lee at al., 2005). The apical cortex localization of aPKC is regulated by Lgl and Pins, and its overexpression induces ectopic neuroblast self-renewal. Moreover, aPKC null mutant resulted in reduced neuroblast number, which indicated that *aPKC* is a neuroblast fate determinant (Lee at al., 2005). A cell fate determinant in the basal cortex of neuroblasts, Prospero, the homeodomain

transcription factor, is directed by Miranda into differentiating GMCs during the asymmetric cell division (Ikeshima-Kataoka et al., 1997). Prospero inhibits neuroblast self-renewal, and mutant Prospero leads GMCs to transform into neuroblasts (Betschinger et al. 2006; Lee et al. 2006). Thus, aPKC and Prospero act in different daughter cells as cell fate determinants.

Numb is a phosphotyrosine binding domain protein (Dho et al., 1998). Similar to Prospero, Numb has basal cortex localization in *Drosophila* neuroblasts and acts as a GMC fate determinant (Lee et al., 2006). In *Drosophila* sensory organ precursor (SOP) cells, Numb also acts as one of the key determinants (Rhyu et al., 1994). As a cytoplasmic adaptor protein, Numb promotes some transmembrane cargo proteins, to undergo endocytosis (Huang et al., 2005). Numb binds to α-Adaptin and polarizes its distribution. α-Adaptin in turn binds to transmembrane proteins and targets them for endocytosis (Berdnik, et al., 2002). This inhibits notch signaling possibly by causing its endocytosis. Numb is asymmetrically segregated into one daughter cell, generating the difference in fate (Berdnik, et al., 2002) .

1.3 Epithelial cell polarity and tumorigenesis

Most human cancers are derived from epithelial tissues and loss of cell-cell adhesion as well as cell polarity is commonly found in epithelial tumours (reviewed by Wodarz and Nathke, 2007). Recent evidence suggests that loss of polarity can directly lead to increased proliferation and tumour-like growths, suggesting a strong connection between epithelial polarity and tumorigenesis (Yamanaka et al., 2006; Plant et al., 2003).

The link between epithelial polarity and tumorigenesis was found by screening for mutations that cause defects in the imaginal discs which are an epithelial tissue (reviewed by Bilder D., 2004). Scrib is a component of the septate junction, a structure similar to the mammalian tight junction. Imaginal discs without Scrib not only lose epithelial polarity but also show cancerous like overgrowth. The same phenotype was found in two other *Drosophila* mutants: Dlg and Lgl (Bissell and Radisky, 2001). Scrib and Dlg colocalise at the basolateral domain of septate junction, directing the polarized sorting of some Lgl-containing vesicles (Bilder and Perrimon, 2000). Loss of this directional-sorting mechanism causes the loss in polarity and the imaginal discs cancerous overgrowth (Bilder and Perrimon, 2000). The human homologue of Lgl, Hugl-1 (Human Lgl1), can rescue of the *Drosophila* Lgl mutation, which suggests that Hugl-1 has a conserved function as the tumour suppressors (Grifoni et al., 2004). The finding of decreased Hugl-1 levels in human colorectal cancer provides further evidence of the putative tumour suppressor function (Schimanski et al., 2005).

Another key regulator of epithelial polarity is the PAR-aPKC complex described above. In addition to Scrib, Dlg and Lgl, the PAR-aPKC complex has multiple links to cancer formation. For example, a human aPKC homologue aPKC iota normally localises in the apical membrane and is absent from the basal membrane in ovarian surface epithelial cells, but in ovarian cancer cells, aPKC iota expression is mislocalised to the cytoplasm and is overexpressed. Moreover, high aPKC iota levels correlate with defects in polarity and poor survival rates (Eder et al., 2005). In addition to ovarian cancer, aPKC iota overexpresssion has been reported in colon

cancer (Murray et al., 2004) and lung cancer (Regala et al., 2005). These findings suggested that aPKC iota is an oncogene.

The PAR-aPKC complex is also associated with tumor suppressors. Phosphatase and tensin homolog (PTEN) is a tumor suppressor protein in human cancer (Rossi and Weissman, 2006). PTEN is crucial for apical-basal polarization. During the polarization, PTEN targets the apical membrane domain to recruit the PAR-aPKC complex (Rossi and Weissman, 2006). Taken together, these studies directly implicate loss of cell polarity in the development of cancer.

The molecular mechanism, linking cell polarity and tumorigenesis, is not clear. One hypothesis is that loss of cell polarity is required for epithelial-mesenchymal transition (EMT), which then promotes cellular motility and invasiveness (Thiery, 2002). Loss of polarity may also allow growth factors and receptors, which are normally separated by the tight junctions in polarized cells, to mediate autocrine cell activation (Vermeer et al., 2003).

In summary, the correct functioning of tumor suppressor proteins and oncogenes is crucial for maintaining cell polarity and preventing tumour formation. This makes it important to understand the mechanisms of differentiation and subsequent maintenance of polarised epithelial cells.

1.4 TGF-beta (TGF-β) and BMP4 pathway

1.4.1 TGF-beta (TGF-β) pathway

One group of proteins which have been linked to epithelial differentiation and tumourogenesis are members of the Transforming Growth Factor β (TGF-β) superfamily. This superfamily is a large group of secreted polypeptide growth factors, characterized by six conserved cysteine residues (Lander et al., 2001). The superfamily includes three subfamilies: the TGF-βs, the activins, and the BMPs (Bone Morphogenetic Proteins). They are defined by sequence similarity and the specific signaling pathways they activate (Attisano L, et al, 2002). The mechanism of the TGF-β superfamily pathway has been well studied. Type I and type II transmembrane serine/threonine kinase receptors, are involved in TGF-β signals transduction. Within a subfamily, these ligands form a homomeric or heteromeric complex that binds to two types of receptors. The type I includes ALK2, ALK3/BMPRIA and ALK6/BMPRIB. The type II consists of BMPRII, ACTRIIA and ACTRIIB (reviewed Massagué and Chen, 2000).

The prevailing view is that TGF-β ligands bind to the type II receptor, and then activates a type I receptor (Ducy and Karsenty, 2000). After the ligand/type II/type I ternary complex formation, the type II receptor phosphorylates the GS domain of the type I receptor, this activates the type I receptor (Wrana et al., 1994). Then, receptor-regulated Smads (R-Smads) were phosphorylated by the type I recepter to initiate cellular responses (Patterson and Padgett, 2000). The R-Smads have two subfamilies: Smad2 and 3 function as R-Smads for TGF-β and activin signaling, whereas Smad1, 5 and 8 relay signaling in response to BMPs (reviewed by Johnsen et

al., 2002). The activated R-Smads in turn form complexes with their adaptor molecule, common-partner Smad4 (Co-Smad) (reviewed in Moustakas, 2002). Inhibitory Smads (I -Smads) are another group of regulatory Smads, which antagonize signaling by R-Smads and Co-Smad, including Smad6 and Smad7 (Heldin et al., 1997). The R-Smad/Co-Smad complexes translocate into the nucleus and regulate target genes transcription. Smads interact with various partners, such as, Fast1, Jun/Fos, Runx, CREBP, NF-kB, β-catenin (reviewed by Shi and Massague, 2003) and regulate a large number of target genes.

1.4.2 BMP and BMP receptors

The BMP pathway initiates the signaling cascade through a heterotetrameric complex of type I (ALK2, 3, 6) and type II (BMPRII) receptors. Subsequent intracellular signalling is mediated via activation of Smads 1, 5 and 8, which collaborate with Smad4, and thus modifies target gene expression (Attisano and Wrana, 2002).

Activin receptor - like kinase-2 (ALK2), known as ACTR-I or TSK7L, was originally thought to be an activin or TGF-β type I receptor, because it binds with the respective type II receptor (Ebner et al. ,1993). However, ALK2 also acts as a type I receptor for BMPs (Miyazono et al., 2005; Tsuchida et al., 2008). Indeed, ALK2 binds to BMP7 and activates BMP-responsive reporters (Macias-Silva et al., 1998). Overexpression of ALK2 in *Xenopus* embryos induced ventral mesoderm like BMP, whereas activin induces dorsal mesoderm formation (Armes and Smith, 1997). Furthermore, a difference between ALK2 and other receptors was found in other species, for example, overexpression of ALK2 inhibited activin-induced cell growth

arrest of B cell hybridoma, whereas ACTR-IB facilitated this processing (Hashimoto et al., 1998). It appears that ALK2 is most likely to act as a BMP receptor.

Activin receptor-like kinase-3 (ALK-3) is another type I receptor for BMP ligands. The extracellular domain of ALK-3 directly binds to BMP2 (Koenig et al. 1994), BMP4 (ten Dijke et al. 1994) and BMP7 (Macias-Silva et al. 1998). ALK-3 can interact with BMPRII, ActRII and ActRIIB (Yamashita et al. 1995). In addition, ALK-3 also forms a heterodimeric complex with ALK-6 (Gilboa et al. 2000). ALK-6 has similar ligand specificities with ALK-3 (Liu et al. 1995). However, the ligand affinities vary between ALK-3 and ALK-6, for example, BMP7 has a higher affinity for ALK-6 than for ALK-3 (Liu et al. 1995; Yamashita et al. 1995). ALK-2, ALK-3 and ALK-6 share the same set of Smad effectors, including Smad1, Smad5, and Smad8 (reviewed by Kawai et al., 2000).

1.5 BMP4 is involved in epithelial differentiation and epithelial tumourigenesis.

Increasing evidence connects BMPs with the differentiation of many types of epithelial cells. Expression of BMP4 is observed in the developing limb, lung, kidney, hair follicle and tooth bud, where inductive interactions occur between the mesenchyme and adjacent epithelium (reviewed by Bitgood and McMahon, 1995). In addition, overexpression of BMP signaling antagonists or knockout of members of the BMP signaling pathway causes epithelial defects. For example, overexpression of Smad7 results in delayed and aberrant hair follicle morphogenesis, causing psoriasis-like skin disorder (Li et al., 2004). Overexpression of Noggin also prevents

hair precursor cells differentiation in epithelial tissues (Plikus et al., 2004). In human intestinal epithelial cells, BMPs promote epithelial differentiation (Kosinski et al., 2007) and in P19 embryonal carcinoma cells treated with BMP4 protein increase the expression of epidermal cytokeratins (Glozak and Rogers, 1996), which is consistant with the role of BMP4 in epithelial formation and may result from enhancement of keratinocyte commitment (Coraux et al.,2003). These findings indicate the significance of BMPs in epithelial differentiation.

BMP signaling has been implicated in tumorigenesis as well. BMP signaling changes the behaviour of a diverse array of cancer cells, causing a range of effects from growth inhibition and apoptosis to influencing metastatic potential (reviewed by Kawamura et al., 2002). In epithelial cancer cells, such as epithelial ovarian cancer (OvCa) cells, BMP4 signaling affects a range of cellular processess including cellular morphology, adhesion, motility and invasion (Brigitte et al., 2007). The findings described above suggest a tight link between BMPs, epithelial differentiation and tumorigenesis.

1.6 BMP4 in early *Xenopus* embryonic development

In addition to epithelial differentiation and tumorigenesis, BMP4 is essential for early embryo development. This has been demonstrated in a number of developmental models, including *Xenopus* embryos, one of the model systems used in this project.

1.6.1 Mesoderm patterning

In *Xenopus*, embryonic mesoderm initially arises from the marginal zone during the pregastrula stage and can be divided into three primary domains: dorsal, ventrolateral, and ventral (reviewed by Vonica and Gumbiner, 2007). The dorsal-ventral axis in *Xenopus* is specified by two important centres: the Nieuwkoop centre and Spemann's organizer (reviewed by Vonica and Gumbiner, 2007).

A role for BMP4 in 'ventralization' is supported by the observation that BMP4 is expressed in the ventral and lateral marginal zones (VMZ and LMZ) of the early gastrula and BMP4 is excluded from the newly formed Spemann's organizer (Schmidt et al., 1995). BMP signaling is active in the ventral and lateral marginal part of the embryo (reviewed by Schohl and Fagotto, 2002). Expression of a dominant-negative BMP4 receptor in isolated animal caps prevented ventralization (Suzuki et al., 1994). In addition, ventral territory expressing genes such as *Vent1*, *Vent 2* are activated by BMP4 (Friedle et al., 1998; Onichtchouk et al., 1996). In parallel, BMP target genes function as efficient repressors of organizer-specific gene expression (Onichtchouk et al., 1998; Shapira et al., 2000). The mutually repressive interactions between the dorsal (organizer) and ventral (BMP) signals and their relative strengths determine the dorsoventral patterning of the embryo (De Robertis and Kuroda, 2004).

1.6.2 Neural specification

Spemann's experiment first established the concept of neural induction, which describes an instructive interaction between the dorsal lip of the blastopore (called the organizer) and the neighbouring ectoderm which induces the nervous system

(reviewed by De Robertis and Kuroda, 2004). Several key neuralizing genes are expressed in the organizer including: *Noggin* (Smith et al., 1993), *Follistatin* (Hemmati-Brivanlou et al., 1994) and *Chordin* (Sasai et al., 1994). Follistatin inhibits BMP7 (Yamashita et al., 1995). *Noggin* and *Chordin* both encode secreted proteins, which bind with high affinity and thereby inactivate BMP4 protein (Zimmerman et al., 1996).

Inhibiting BMP pathway in *Xenopus* embryos generates ectopic neural tissue (Hemmati-Brivanlou and Melton, 1994). Results from dissociated animal cap showed that inhibiting cell-cell signaling leads to the neural formation (Wilson and Hemmati-Brivanlou, 1995) and based on these experiments the default model for neural specification was proposed. The model said that the neural ectoderm forms when the BMP pathway is inhibited (Wilson and Hemmati-Brivanlou, 1995). However, this model was challenged by recent results. For example, two key molecules secreted by the organizer with neuralizing activity, Chordin and Noggin, can not induce neural formation when overexpressed with a dominant-negative FGF (Fibroblast Growth Factor) receptor (Launay et al., 1996). Overexpressing Smad6, which inhibits BMP signaling by acting on Smad1, in the ventral ectoderm was sufficient to repress epidermal formation but failed to promote neural induction (Delaune et al., 2005). Those results indicated neural specification requires additional signals. Moreover, the most likely factor is FGF.

Recent studies in *Xenopus*, zebrafish and chick, confirmed the importance of FGF in neural induction. Both the activation of FGF signaling and the repression of BMP signaling are necessary for the neural fate specification (Delaune et al., 2005). In

chick and zebrafish, FGF signaling inhibits *Bmp* gene expression (Wilson et al., 2000). Recent findings indicate FGF signaling can phosphorylate the linker region in the middle of the BMP effector Smad1, to induce neurogenesis via inhibiting BMP signaling; whereas BMP signaling activates Smad1 by phosphorylating its C-terminal domain (De Robertis and Kuroda, 2004). In this manner, FGF may promote neural induction, both directly and by inhibiting BMP signaling (Figure 3).

1.6.3 Epidermal specification

The dissociated animal cap experiments indicated that short range cell-to-cell signaling is required for epidermal formation (Wilson and Hemmati-Brivanlou, 1995). BMP4 can direct *Xenopus* dispersed gastrula ectoderm to form epidermis instead of neural tissue (Hemmati-Brivanlou, and Melton, 1994). Their further report suggested BMP4 is an epidermal inducer (Wilson and Hemmati-Brivanlou, 1995; Hemmati-Brivanlou and Melton, 1997). It also became clear that neural/ epidermal specification was not a simple binary choice. By using dispersed *Xenopus* animal cap cells, distinct responses to different BMP4 concentrations were observed (Hemmati-Brivanlou and Melton, 1997). Cement gland was induced at lower concentrations and epidermis at high concentrations (Wilson and Hemmati-Brivanlou, 1995; Hemmati-Brivanlou and Melton, 1997). Without BMP4, neural tissue will form (Wilson and Hemmati-Brivanlou, 1995; Hemmati-Brivanlou and Melton, 1997). The formation of three different cells population suggested BMP specifies the cell fates, including both neural and epidermis, in a dosage-dependent manner.

The concentration dependent manner of active BMP4 was also noticed at the level of

transcription by Dosch and the colleagues (Dosch et al., 1997). At low concentration of BMP4, a marker of the dorsolateral domain *Myf*-5 shifts into the organizer; and the ventral marker *Vent-1* expression expands (Dosch et al., 1997). With increasing dosage of BMP4, *Myf-5* and *Chordin* expression are completely inhibited. Noggin activity has a gradient, with the opposite direction to BMP4 (Dosch et al., 1997). Low doses of Noggin activate *Myf-5* on the ventral side and inhibit *Vent-1* expression. High doses repressed *Myf-5* and *Vent-2* and produced the radial Chordin expression (Dosch et al., 1997). Thus, the dose-dependent expression of Noggin functions to create graded BMP4 activity.

Noggin and Chordin can bind BMP2 and BMP4 (Zimmerman et al., 1996). Furthermore, dominant negative BMP7 ligand acts as a neural inducer (Hawley et al., 1995). This data raised the possibility that other BMPs are also involved in epidermal induction. It was showed that secreted BMP2 and BMP7 can mediate epidermal specification like BMP4 (Suzuki et al., 1997a). Their results also suggested that epidermal induction could be mediated by heterodimerization between BMP2/4/7 ligands (Suzuki et al., 1997a; Suzuki et al., 1997b).

Summarizing the details described above, is a neural/epidermal specification model. Neural ectoderm and epidermis are positioned at blastula stages in response to BMP, FGF and Nodal-related pathways. BMP inhibitors (Chordin, Noggin) and FGF signaling repress *Bmp4* to induce a BMP-free region in the ectoderm, where neural tissue forms (reviewed by Delaune et al., 2005). In contrast, epidermis forms where BMP functions are maintained (reviewed by Delaune et al., 2005).

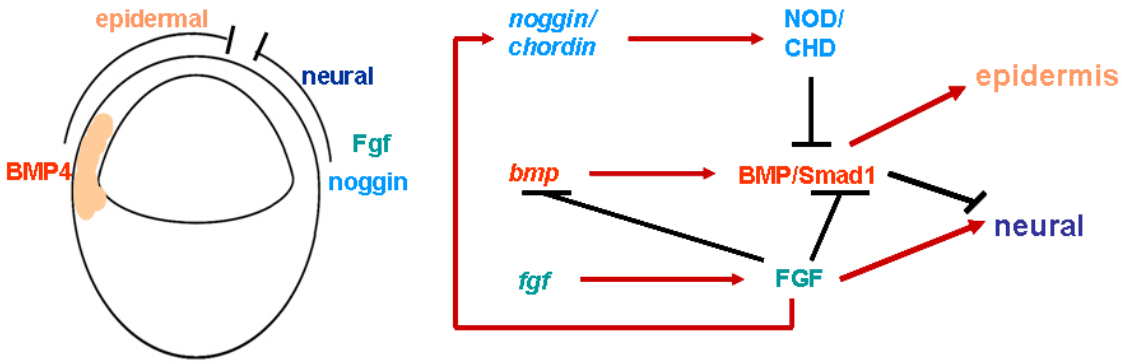


Fig 3 Neural/epidermal specification model in *Xenopus* (modified from Stern, 2007). Neural ectoderm and epidermis are positioned in response to the BMP, FGF and Nodal-related pathways. BMP inhibitors (Chordin, Noggin) and FGF signaling repress Bmp4 to induce a BMP-free region in the ectoderm, where neural tissue forms (reviewed by Delaune et al., 2005). In contrast, epidermis forms where BMP functions are maintained (reviewed by Delaune et al., 2005).

1.6.4 BMP activates a number of transcription factors during epidermal induction

To understand how BMP signaling controls epidermal development, it is vital to identify the downstream targets in the BMP cascade and BMP mediators. This includes the the immediate early response genes which are direct targets and genes which are activated indirectly.

1.6.4.1 *Msx* genes

Immediate early response genes in the epidermis include members of the Msx family of transcription factors. The *Msx* family includes the vertebrate homologues of *Drosophila Msh* (muscle segment homeobox) gene (Davidson, 1995). In *Xenopus*, chick and mouse, results have suggested that *Msx* family members can mediate some functions of BMPs in regulating epidermal induction, and facial tissue (Davidson, 1995).

The link between *Msx* and *Xenopus* epidermis was revealed by finding *Msx1* expression in the ventral ectoderm and mesoderm (Keller, 1991). In fact, overexpression of *Msx1* in early *Xenopus* embryos leads to ventralization. In dissociated ectodermal explants, *Msx1* can induce epidermis and inhibit neural differentiation, like BMP4 (Suzuki et al., 1997c). Suzuki‘s work also demonstrated that *Msx1* is induced by BMP signaling in the early amphibian ectoderm (Suzuki et al., 1997c), both BMP ligands and an activated BMP receptor induce *Msx1* expression. However, the induction does not require *de novo* protein synthesis (Suzuki et al., 1997c). Afterward, it was reported that Msx1 has several Smad-binding sites in its promoter region (Binato et al., 2005). These observations strongly suggest that *Msx1* acts as an immediate early response gene to BMP4 in *Xenopus* embryos, to mediate BMP signaling in epidermal induction, and inhibit of neural differentiation.

However, Khadka’s research challenged the hypothesis that Msx functions in epidermal induction. By using antisense morpholino oligonucleotides (MOs), they showed that blocking *Msx* expression in *Xenopus* does not affect epidermal development (Khadka et al., 2006). The result indicates Msx1 and 2 are not required for epidermial development, which might be explained by redundancy with other factors such as Vent-2 and AP-2.

1.6.4.2 Vent-2

Another candidate for a BMP4 immediate early response gene is *Vent-2*, which is expressed in the marginal zone of the early *Xenopus* gastrula, excluding the organizer

region. Vent-2 expression overlaps with BMP4. Furthermore, *Bmp4* induces expression of *Vent-2* and vice versa (Onichtchouk et al., 1996). This result indicated the possibility that Vent-2 and BMP4 are required for each others' expression. Overexpressing *Vent-2* can rescue the dorsalization by a dominant negative BMP4 receptor (Onichtchouk et al., 1996). Moreover, Vent-2 expression does not require *de novo* protein synthesis, like Msx1 (Onichtchouk et al., 1996). There is direct interaction between Smad1 and Vent-2 (Henningfeld et al., 2002). These findings indicate *Vent-2* is another immediate early response gene of BMP4. This puts *Vent-2* directly downstream of BMP4 signaling and suggests *Vent-2* may play an essential role in BMPs regulated cell fate specification.

1.6.4.3 Ap-2 and Dlx

The *Xenopus* homolog of the mammalian transcription factor AP-2α (Ap-2) is an essential factor for the embryonic type I keratin expression, which is an early marker for ventral/ epidermal specification (Snape et al., 1991). More recently, *Ap-2* was found to be regulated by the BMP pathway. Luo's research confirmed *Ap-2* expression is BMP-dependent, and directly controls Keratin expression. Furthermore, Ap-2 is required for epidermis-specific gene expression (Luo et al., 2002).

A member of the Distal-less (*Dlx*) family of *Xenopus*, *Dlx3* has been implicated in the control of epidermal cell differentiation under positive regulation of BMP signalling in *Xenopus* embryos (Luo et al., 2001). *Dlx5* was reported in defining the rostral limit of the neural plate (Papalopulu and Kintner, 1993). Ectopic Ap-2 can restore keratin and *Dlx5* gene expression in neuralized ectoderm (Luo et al., 2002), suggesting that *Dlx3/5* is part of the epidermal regulation network and functions downstream of *Ap-2*.

Ap-2 and *Dlx3/5* are both essential for epidermal specification. However, BMP4 induction of AP-2 and Dlx3 is dependent on *de novo* protein synthesis (Luo et al., 2001; Luo et al., 2002). Moreover, *Ap-2* can bind directly with the keratin promoter (Tao et al., 2005). Those finding indicated that Ap-2 and Dlx3/5 are downstream of the immediate early response genes of BMP4 and may directly promote epithelial structure genes expression.

1.6.4.4 Grh family

Genes at the bottom of the BMP4 cascade which carry out the functions of BMP signaling in different cell types are as important as the immediate early response genes. Research in *Xenopus* and other model species indicates members of the Grainyhead family may function in this way.

Drosophila Grainyhead protein (*Grh*, also known as NTF-1 or Elf1) is a sequence-specific DNA-binding protein that defines a family of transcription factors conserved from *Drosophila* to mammals (Bray and Kafatos, 1991). In *Drosophila*, Grh is expressed in several tissues, where it is involved in different developmental programs (Bray and Kafatos, 1991). Six members of Grh family have been identified in mammals (reviewed by Parekh et al., 2004). Intriguingly, many Grh family members are expressed in epithelial tissues and have roles in epithelial morphogenesis (Ting et al., 2005). Notably, Tao's research provides the first evidence that *Xenopus* grainyhead-like 1 (*Grhl1*) is important for epidermal ontogeny. The epidermal specific transcription factor is regulated in a BMP4 dependent manner, but is not directly

regulated by BMP4, which like Ap-2 and Dlx3/5 (Tao et al., 2005). They proposed the following model: BMP signalling directly activates Vent2 and Msx1. Then Vent2 and Msx1 regulate the epidermal specific transcription factor Grhl1, which can directly regulate epidermal structural genes such as the cytokeratin (*Keratin*) (Tao et al., 2005).

Another member of the Grh family, Grhl3, can promote *Keratin* expression in the superficial layer of *Xenopus* epidermis (Chalmers et al., 2006). This result suggests that Grhl3 may work like Grhl1 as a BMP4 effector. Combinating this data suggests that Grhl family proteins maybe located at the end of the cascade of BMP4 epidermal specification pathway.

Thus, a model for epidermal differentiation has been proposed (Fig. 4). In epidermal development, BMP binds to its cognate receptor and activates expression of immediate early response genes *Msx1* and maybe *Vent2* (Onichtchouk et al., 1996; Suzuki et al., 1997c; Wilson et al., 1997). In turn, direct targets modulate other transcriptional factors, such as Ap-2 and Dlx-3, and Grhl family in ectodermal cells (Luo et al., 2001; Luo et al., 2002). *Grhl1* induces *Ap-2* and *Dlx3* expression in dissociated animal explant cells (Tao et al., 2005). This observation suggests a positive feedback loop (Green, 2002). Finally, those transcription factors promote the epidermal differentiation in the early embryos.

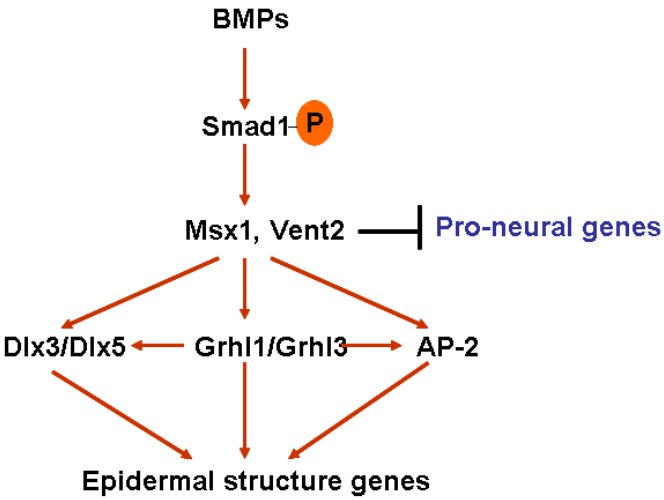


Fig 4 The epidermal regulatory network (modified from Heasman J, 2006). BMP signalling directly activates *Vent2* and *Msx1*. Then *Vent2* and *Msx1* regulate the epidermal specific transcription factors Grhl1/Grhl3/AP-2/Dlx3/Dlx5, which can directly regulate epidermal structural genes such as *Keratin* (Tao et al., 2005; Chalmers et al., 2006).

1.7 Embryonic stem cells are a good model for studying mouse embryo development

Originating from the mammalian inner cell mass (ICM) of the blastocyst, embryonic stem cells (ESCs) can differentiate into the three germ layers, ectoderm, mesoderm and endodermal lineages (reviewed by Takahashi and Yamanaka, 2006). The fate of these three germ layers is different. The endoderm forms the epithelial lining of respiratory and gastrointestinal tract. The mesoderm gives rise to the cardiovascular system, muscle, connective tissues, vessels, and skeleton. The ectoderm forms the epidermis, nervous system, neural crest cells and derivatives (reviewed by Takahashi and Yamanaka, 2006). This ability of mouse embryonic stem cells (mESCs) to develop into any embryonic cell makes them a good model system to study developmental mechanisms. It is also becoming clear that mESCs can form extraembryonic trophectoderm under certain conditions (reviewed in 1.8.3.3). This makes it possible to study formation of the first epithelium, the trophectoderm using

mESCs, something which is carried out in Chapter V.

In addition to differentiating, a mESC can produce more pluripotent cells, which is called self-renewal. The precise balance between self-renewal and differentiation of stem cells is essential for processes ranging from embryonic development and organogenesis to tissue regeneration (reviewed by Weissman, 2000). If the balance is not properly controlled, normal homeostasis will be destroyed; furthermore, cancer and tissue defects may emerge (Weissman, 2000). Thus, identifying the molecular mechanism regulating the balance between self-renewal and differentiation is one of the biggest issues in stem cell research. This issue is relevant to understanding the process of differentiation as factors which promote differentiation are likely to work against those that promote self-renewal.

1.7.1 mESCs self-renewal and differentiation: the core regulatory network

The molecular mechanisms controlling the balance between pluripotency and differentiation are starting to be understood. An emerging concept is that a core regulatory network of transcription factors forms a well-regulated circuit. The autoregulatory core promotes the genes controlling self-renewal while repressing differentiation. The core regulatory network includes *Oct3/4*, *Sox2*, and *Nanog* (reviewed by Chickarmane et al., 2006).

The POU transcription factor Oct3/4 (Octamer-4) (also known as Pou5f1: POU domain class 5 transcription factor 1) participates in ES cell fate regulation (Okamoto

et al., 1990). The expression of Oct3/4 correlates with an undifferentiated phenotype (Scholer et al., 1990). *In vitro*, the overexpression of Oct3/4 promotes primitive endoderm and mesoderm differentiation (Niwa et al., 2000). In contrast, reducing Oct3/4 expression in mESCs results in loss of pluripotency, and differentiation into the trophectoderm lineage (Niwa et al., 2000). Thus, a precise level of Oct3/4 is required for mESCs to remain in an undifferentiated stage.

A range of genes downstream of Oct3/4 have been identified (Bortvin et al., 2003), including *Sox2* and *Nanog*. The Sox2 (SRY-related HMG box 2) transcription factor is expressed in pluripotent stem cells and is involved in maintaining pluripotency. Sox2 collaborates with Oct3/4 and Nanog through a coordinated transcriptional program (Okamoto et al., 1990).

Nanog is another important protein, downstream of Oct3/4 that regulates mESC self-renewal (reviewed in Kuroda et al., 2005; Rodda et al., 2005). A homeodomain-bearing protein, Nanog is specifically expressed in pluripotent cells of mouse, monkey and human (Pan and Thomson, 2007). The regulatory region upstream of the transcriptional start site of *Nanog* gene contains OCT and SOX responsive elements and Oct3/4 and Sox2 are the major transcription factors that bind to the *Nanog* promoter to promote *Nanog* transcription (Rodda et al., 2005; Kuroda et al., 2005).

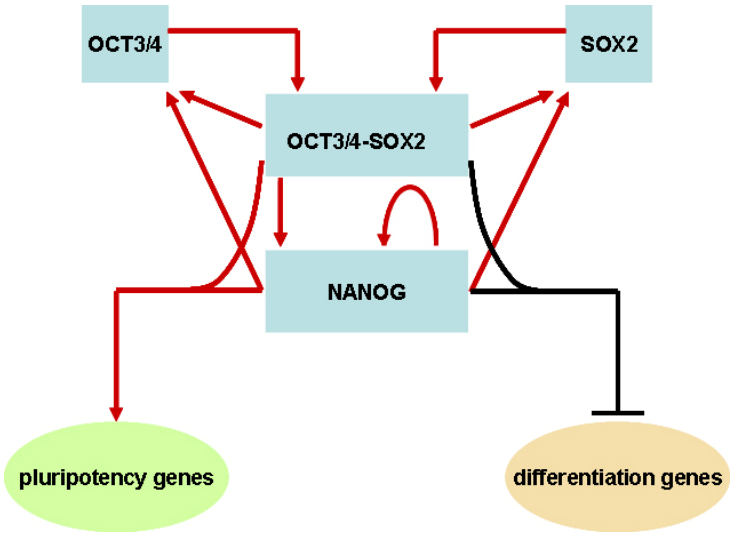


Fig 5 Oct3/4, Sox2 and Nanog form a autoregulatory core (modified from Chickarmane et al., 2006). The transcription-factor core promotes pluripotency genes expression but inhibits differentiation genes, thus maintains self-renewal while represses differentiation.

However, conditional gene knockout in mESCs indicates that *Nanog* is not an essential factor for pluripotency propagation (Chambers et al., 2007). Thus, Nanog may not work in the same way as Oct3/4 and Sox2. Nanog is expressed weakly, or is not expressed in incompletely reprogrammed cells, so that the endogenous pluripotent transcriptional program cannot finish (Sridharan et al., 2009). Moreover, after mESC fusion, Nanog promotes the transfer of pluripotency (Silva et al., 2006). Pluripotency does not develop without Nanog, and the ICM is trapped in an indeterminate state (Silva et al., 2009). Thus, Nanog works as a gateway for cells to reach, but not maintain, pluripotency. A model summarizing the regulation of pluripotency is presented in figure 5.

1.7.2 Extrinsic regulators of self renewal

mESCs can be expanded without losing their pluripotency in well controlled *in vitro* culture conditions. For example, they can be co-cultured with serum and heterologous

feeder cells (Niwa et al., 1998), which suggests that the maintenance of mESCs is dependent on feeder cell-derived growth factors.

Leukemia inhibitory factor (LIF) is one of the most investigated factors produced by mouse embryonic fibroblast (Bard and Ross, 1991). LIF signals through STAT3 (Signal Transducer and Activator of Transcription-3) to block mesoderm and endoderm differentiation (Ying et al., 2003a). However, without serum, LIF is not sufficient to maintain mESCs self-renewal, and the cells differentiate to a neural fate. Addition of LIF and BMP4 is able to maintain mESCs self-renewal and pluripotency (Ying et al., 2003b). BMP4 activates the Inhibitor of Differentiation (Id) family to block entry into neural lineages (Ying et al., 2003a). In summary, both intrinsic and extrinsic factors contribute to maintaining self renewal in ESCs.

1.8 The first epithelium in vertebrate development

The first epithelium in vertebrate development has been named the primary superficial epithelium (PSE) (Sabel et al., 2009) and is refered as the superficial layer in *Xenopus* (reviewed by Chalmers et al., 2002), the enveloping layer (EVL) in zebrafish (Kimmel et al., 1995), and the trophectoderm in mammals (Yamanaka et al., 2006).

1.8.1 Superficial and deep cells in *Xenopus* embryos

1.8.1.1 Formation of superficial and deep cells in *Xenopus* embryos

In *Xenopus*, superficial and deep cells are generated during blastula stages by asymmetric cell division. Chalmers and colleagues revealed that all blastomeres are

superficial cells before the 32-cell stage of *Xenopus* development, but the generation of the first deep cell then occurs by perpendicular divisions (Chalmers et al., 2003). During the perpendicular division, the mitotic spindle aligns with the axis of apical-basolateral polarity, perpendicular to the surface of the embryo, so that it produces one outer superficial cell and one inner deep cell (Chalmers et al., 2003). These divisions occur throughout blastula stages and gradually generate the deep cells. Further work suggested that the perpendicular divisions may be regulated by the cell shape (Strauss et al., 2006).

1.8.1.2 Polarity of the *Xenopus* superficial and deep cells

The superficial cells establish an epithelial layer, with polarised apical and basolateral membrane domains and tight junctions (reviewed by Eckert and Fleming, 2008). This layer has a high-resistance seal which comes from the tight junctions of the superficial layer (Regen and Steinhardt, 1986). Interestingly, apical-basolateral polarity is established as early as the 2 cell stage and cells can establish polarity even without cell-cell contact (Muller and Hausen, 1995). These findings suggested that the polarity determinant comes from an intrinsic factor localised in the egg membrane. In *Xenopus* blastomeres, aPKC (iota and zeta) localise in the apical domain, moreover overexpression of aPKC results in expansion of the apical membrane (Chalmers et al., 2003; Chalmers et al., 2005). During the asymmetric cell division, aPKC (iota and zeta) asymmetrically localise in the apical membrane of cells (Chalmers et al., 2002). The results suggest that aPKC acts as the polarity determinant in *Xenopus* and may also regulate cell fate.

Overexpression of *Lgl* resulted in the loss of TJs in the outer epithelial layers

(Chalmers et al., 2005). This is the same as the phenotype produced by expressing the dominant-negative aPKC. Moreover, expressing *Lgl* rescued the aPKC-induced expansion of the apical membrane. Expressing aPKC also inhibited the localisation of Lgl. These results suggest that mutual inhibition between aPKC and Lgl defines the apical and basolateral domain respectively in *Xenopus*, a similar mechanism to that described for other types of epithelial cells, for example, MDCK cells (Madin Darby Canine Kidney) (Suzuki et al., 2004). Apical-basolateral polarity is only found in the outer superficial cells and not the underlying deep cells (Chalmers et al., 2003). Consistent with this deep cells do not have cortical aPKC so LGL localises throught the membrane (Chalmers et al., 2005).

1.8.1.3 Fate regulation in superficial and deep cells in Xenopus

In *Xenopus* the fate of the superficial cells is complicated and depends on their position in the embryo. The superficial cells in the vegetal and marginal zones form the lining of the archenteron which will incorporate into the gut tube during later development (Chalmers and Slack, 2000) and little is known about the development of these cells.

In the animal hemisphere, the dorsal side of the superficial layer will form the superficial layer of the neural plate. This layer does not form primary neurons, which originate from the deep cells but becomes secondary neural precursors (reviewed by Chalmers et al., 2002). Overexpressed *Noggin* induces superficial layers to express the neural marker Sox3 indicating that superficial cells have the potency for neural induction (Chalmers et al., 2002). However, isolated superficial and deep layers have intrinsic differences in their response to induction of neuronal differentiation

(Chalmers et al., 2002). Endogenous signals from the organizer and transcription factors which promote neurogenesis produce a stronger induction in deep cells than in superficial cells (Chalmers et al., 2002). The difference in competence may explain the difference in neurogenesis seen between the two layers. The difference in competence could be caused because the superficial layer expresses the transcription factor ESR6e which can inhibit neural differentiation. ESR6e overexpression inhibits primary neurogenesis (Chalmers et al., 2002). Recent work showed that neuronal differentiation in the deep cells is promoted by Par-1, which is inhibited by aPKC in the superficial cells (Ossipova et al, 2007). The mechanism involves the phosphorylation of mind bomb, which represses notch signaling and allows neurogenesis to occur (Ossipova et al, 2009).

At the ventral side of the animal hemisphere, superficial cells give rise to the outer layer of the epidermis and provide a simple protective outer layer for the whole embryo, which may be then shed in later development (Jones and Woodland, 1986; Furlow et al., 1997). The deep cells of the epidermis either remain undifferentiated at early stages or form ciliated and non-ciliated cells later (Deblandre et al., 1999; Drysdale and Elinson, 1992). Ciliated cells intercalate into the superficial cells and finally form cells with motile cilia in the *Xenopus* skin. The development of the ciliated cells is controlled by Notch and lateral inhibition (Deblandre et al., 1999).

The difference in fate between the two cell types is established by late blastula stages when the superficial cells begin to express differentiation markers (reviewed by Chalmers et al., 2002; Chalmers et al., 2006). Recently, the transcription factor grainyhead-like 3 (Grhl3) was revealed to be expressed in the superficial layer of the epidermis and be able to promote superficial cell specification and switch off

deep-cell gene expression (Chalmers et al., 2006). These findings strongly suggest that Grhl3 is a key regulator of superficial cell identity. Another transcription factor which is involved in development of the superficial layer of the epidermis is interferon regulatory factor 6 (Irf6) (Sabel et al., 2009). Depletion of Irf6 results in *Xenopus* superficial epithelium disruption. Moreover, *ESR6e* and *Grhl3* expression are slightly downregulated by Irf6 depletion (Sabel et al., 2009). These results suggest possible crosstalk between different superficial fate determinants.

In summary the superficial layer of the *Xenopus* epidermis provides a good model, to investigate what promotes differentiation of the first vertebrate epithelium and it will be used in this project.

1.8.2 Enveloping layer and deep cells in Zebrafish

Zebrafish embryo forms an epithelial monolayer known as the enveloping layer (EVL). The EVL surrounds the entire embryo by gastrula stages and gives rise to the periderm which is shed later in development (Kimmel et al., 1995). The deep layers (DEL) give rise to the cells that will later form the embryo (Sagerström et al., 2005).

The layers in Zebrafish and the layers of the *Xenopus* embryo have a number of similarities. The EVL has TJs, which require the scaffolding protein ZO-3 function, losing ZO-3 results in abnormal embryonic development (Kiener et al, 2008). EVL cell fate, like *Xenopus* superficial cell fate, is specified by midblastula stages (Sagerström et al., 2005). Interestingly, injection of putative dominant negative IRF6

in Zebrafish embryos results in EVL markers, such as keratins, being lost. The results indicate that Irf6 is required for EVL cell fate like it is for *Xenopus* superficial cell fate (Sabel et al., 2009). Deep cells in Zebrafish do not have polarity, and they begin to be formed by orientated cell divisions which, like in *Xenopus*, start at the 32 cell stage (Kimmel and Law, 1985).

1.8.3 Trophoblast and ICM in mouse

The preimplantation mouse embryo consists of two distinct cell types: the trophectoderm (TE) and the ICM. The TE is the outside tissue layer of the early embryo and differentiates into the trophoblast lineage (reviewed by Rossant and Cross, 2001) which produces cells of the placenta. For example, syncytiotrophoblasts grow into the endometrial stroma of uterine tissue and secrete hormones during pregnancy (Rossant and Cross, 2001). Trophoblast giant cells are large polyploid cells producing essential growth factors and hormones for embryonic growth (Cross et al. 2002). Spongiotrophoblasts form the middle layer of the placenta (Cross et al. 2002). The ICM is under the trophectoderm and is the precursor of the embryo (Rossant and Cross, 2001).

1.8.3.1 Formation of TE and ICM

The TE and ICM are formed at the eight-cell stage. Similar to *Xenopus* and Zebrafish superficial polarized cells divide asymmetrically to produce one polar and one inner apolar cell or divide symmetrically to produce two polar daughter cells (reviewed by Jedrusik et al., 2008). The cells without polarity later form the ICM (reviewed by

Jedrusik et al., 2008).

1.8.3.2 Polarity of the trophectoderm cells

Although transcript analysis indicated that most TJs mRNA are found in both TE and ICM the TJ proteins only assemble in the TE lineage at the apical contact of blastomeres (Fleming et al., 2004). This begins at the eight cell stage. One mechanism restricting TJ formation to the outer TE cells of the blastocyst is the pattern of cell contact. Asymmetric cell contacts induce epithelial polarization in the outer TE but in the ICM symmetric contacts suppress polarization (Eckert et al., 2004; Fleming et al., 2004).

Analysis of TJ biogenesis revealed that TJ proteins are organized in a stepwise manner, a process which may be regulated by PKC (reviewed in Eckert and Fleming, 2008). PKCs regulate TJs expression at the RNA level, directly or indirectly (Leotlela et al., 2006). PKC isoforms partially colocalise with the ZO-1 at the initial stage of TJs assembly (Eckert et al., 2004). Moreover, PKCδ and ζ also contribute to blastocoel formation, which requires functioning TJs (Eckert et al., 2004). Those finding indicate that PKC is involved in polarity establishment in TE development.

1.8.3.3 Regulation of TE/ICM fate

Several transcription factors are involved in regulating trophoblast cell fate (Figure 6). Niwa and the colleagues found that decreased expression of Oct3/4 (Niwa et al. 2000) promoted trophectoderm differentiation. They got identical results by overexpressing

the transcription factor Cdx2 (Niwa et al. 2005). Cdx2 is required for TE formation, which was shown by investigating trophoblast cell propagation in a *Cdx2*-knockout mESC line (Niwa et al. 2005). mESCs can be derived from Cdx2-null embryos (Chawengsaksophak et al., 2004), but the embryos do not form TE (Strumpf et al., 2005). Thus, Cdx2 is essential for the maintenance of TE. On the other hand, Oct4 is expressed abundantly in the external cells of Cdx2-null embryos, indicating that Cdx2 is necessary to repress the expression of Oct4 in TE (Strumpf et al., 2005; Niwa et al., 2005).

A T-box transcription factor Eomesodermin (Eomes), Eomes is expressed in the TE lineage (Ciruna and Rossant, 1999; Russ et al., 2000) and Cdx2-null embryos have remarkably decreased Eomes expression (Strumpf et al., 2005; Ralston and Rossant, 2008). Eomes knockouts demonstrate that it is also required for TE formation (Strumpf et al., 2005). Same with Cdx2-null embryos, Eomes-null embryos are unable to generate trophoblast giant cells and trophoblast cell lines (Russ et al., 2000; Strumpf et al., 2005). However, they display the correct expression patterns of Oct4 and Cdx2 (Strumpf et al., 2005). These results indicate that Eomes is a target of Cdx2. But weak expression of Eomes in Cdx2-null embryos also suggests a Cdx2-independent mechanism for Eomes expression.

Tead4 is a TEA domain transcription factor which is expressed in the blastocyst (Nishioka et al., 2008). Tead4-null embryos exhibit defects specifically in TE (Yagi et al., 2007; Nishioka et al., 2008). The phenotype of Tead4-null embryos is similar to Cdx2-null-embryos, but it is more severe. Notably, Cdx2 is absent while Oct4 and Nanog are expressed in external cells at late blastocyst stage (Yagi et al., 2007;

Nishioka et al., 2008), which indicates the *Cdx2* gene may be regulated by *Tead4*. *Eomes* expression is also absent in Tead4-null embryos (Nishioka et al., 2008), suggesting that Tead4 also regulates Eomes. Gata3 is another Tead4 regulated transcription factor. Unlike Eomes, Gata3 can induce TE fate even without *Cdx2* (Ralston et al., 2010), suggesting Gata3 acts via a Cdx2-parallel pathway.

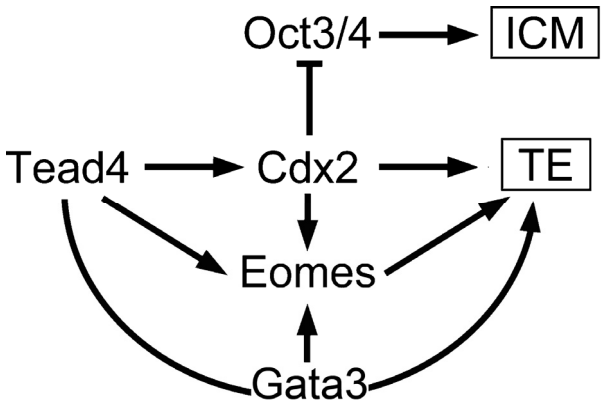


Fig 6 Tead4 regulates Cdx2 and Eomes expression to direct TE formation (modified from Nishioka et al., 2008). Cdx2, Eomes and Gata3 are regulated by Tead4 and they promote TE specification. The transcription factors, at least Cdx2, inhibit Oct3/4, which prevents ICM specification.

Several observations raise the possibility that TE fate specification transcription factors are down stream of cell polarity. Relocating apolar inner cells to the outside results in the cells polarizing and acquiring TE fate (reviewed by Yamanaka et al., 2006). However, it is not known how cell polarity regulates TE fate. Yes-associated protein 1 (YAP1, Yap hereafter), regulates Tead4 activity in a position-dependent manner (Nishioka et al, 2009). In ICM cells, hippo signalling inactivates YAP1 by phosphorylation via Lats1/2, so that YAP1 was released from nuclei, which results in Tead4 activity inhibition. In TE cells YAP1 maintains the nuclei enrichment, which allows Tead4 activity to regulate TE specification (Nishioka et al, 2009). This might provide a link between polarity and transcription factors. Down regulation of aPKC and PAR3 results in increased contribution to the ICM fate in the preimplantation

mouse embryo, suggesting that these proteins could also be involved in regulating cell fate (Plusa et al. 2005).

Similarities in the polarization and asymmetric cell division indicate that there may be conserved mechanisms which promote differentiation of the PSE in different vertebrates. However, it is remains unknown what promotes differentiation of this epithelium and to what extent there is conservation of cell fate regulation between different vertebrate species.

1.9 Aim

The project aims to identify the factors promoting differentiation of the first vertebrate epithelium. This will involve testing potential candidates to establish if they can promote superficial cell fate in *Xenopus* embryos, then taking the factors to establish if they are also able to promote superficial cell fate in other vertebrate such as mouse. To do this mESCs will be used as an *in vitro* model of mouse embryos.

2 Chapter II Materials and methods

2.1 Molecule biology

2.1.1 Plasmids preparation

Plasmid preparations were carried by using a QIAGEN Plasmid Maxi Kit (QIAGEN, 12163) or a Wizard Plus SV Miniprep DNA purification system (Promega, A1330), protocols following manufacturers instructions.

2.1.2 Linearising DNA for making RNA

10 μg of plasmid DNA was linearised in a 100μl reaction with an appropriate restriction enzyme (see Table 5) by incubation at 37 °C for at least 2 hours. 5μl of 25mg/ml proteinase K (Roche, 03115879001) and 6μl of 10% SDS (UltraPure™ Sodium Dodecyl Sulfate (SDS), Invitrogen, 15525017) were added to digest proteins by incubation for 30 minutes at 50^{o}C. The volume was made up to 400μl with dH_2O and extracted twice with an equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) (Sigma, P3803). The tube was spun down at 12,000 rpm for 5 minutes and the aqueous phase (top phase) was removed to a new eppendorf tube. The supernatant was then extracted twice with an equal volume of chloroform, spun down and the aqueous (top) phase removed to a new eppendorf. 1/10th volume of 3M NaOAc (pH 5.2) and 2x volumes of ethanol were added and the mix stored at -20^{o}C for at least 20 minutes to help DNA precipitation. The tube was spun for 15 minutes. The pellet was washed with 150μl 70% ethanol and resuspended in 10 μl DEPC-H_2O, 1ul mixture was used to check digestion.

2.1.3 Preparing RNA for injection

Capped sense RNA for injection was transcribed with the Ambion mMessage mMachine kit (Ambion, AM1340 and AM1340). 3µl dH2O (nuclease free from kit), 2µl 10X reaction buffer, 10µl 2X ribonucleotide mix (NTP/CAP), 3µl 1µg/µl linearized template DNA (described above), and 2µl 10X enzyme mix were added and incubated for 2 hours at 37 ^{o}C. 1µl RNase-free DNase I (Roche, 04716728001) was added and incubated for 15 minutes at 37 ^{o}C to get rid of the DNA. The reaction was stopped by adding 115µl nuclease free dH_2O and 15µl ammonium acetate stop solution and extracted twice with an equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) (Sigma, P3803). The tube was spun and the aqueous phase (top phase) removed to a new eppendorf tube. After extraction with equal volume of chloroform twice, the RNA was precipitated with an equal volume of isopropanol, at -20 ^{o}C for 20 minutes. The tube was then spun down for 15 minutes to collect pellet. The pellet was then washed with 150µl 70% ethanol (DEPC-H_2O) and resuspended in 12.5µl nuclease free dH_2O, vortexed for approx 30s then heated for 30s at 80 ^{o}C, this was repeated then the RNA was vortexed 30s and chilled on ice. The RNA was quantitated by both electrophoresis using a fresh gel under EPI Chemi II darkroom (UVP, USA), and by reading the absorbance at OD260 with BioPhotometer Plus (Eppendorf AG, German). RNA was stored as 1µl aliquots at -70 ^{o}C.

The following amounts of RNA were injected: 1ng *aPKC*, 1ng *CAAX-aPKC*, 0.5ng *Notch-ICD*, 1 ng *Bmp4*, 1 ng *GFP*, 1 ng *Grhl3*, 0.1 ng *Noggin*, 1ng *GFP-Lgl*, 0.5ng *Alk2*. As a lineage tracer, 0.5 ng β-galactosidase (*lacZ*) RNA was coinjected with the experimental RNA, as previously described (Detrick et al. 1990; Bourguignon et al. 1998).

2.1.4 Making DIG/FITC labelled *In situ* probes

The RNA probe transcription reaction contained the following: 10ul 5x transcription buffer, 5 ul DTT, 3µl RNA polymerase (T3, T7 or SP6) (see Table 5), 10µl 2.5mM NTP-dig mix (Ribonucleoside Triphosphate Set Lithium salts, Roche, 11277057001), 3 µl 1µg/µl linearized template DNA (DNA was linearised as above), 0.5µl 40u/µl RNase inhibitor (Roche, 11119915001) and 18.5 ul DEPC-H_2O. The reaction was incubated for 2 hours at 37^oC. 1µl 1mg/ml RNase free DNase1 (Roche, 04716728001) was added and incubated for 20 minutes at 37^oC to get rid of DNA fragments. 25ul LiCl (Ambion mMessage mMachine kit) was added to precipitate RNA at -20 ^oC for at least 2 hours, then spun down at 13,000 rpm for 20 minutes to collect pellet. The pellet was washed in 70 % ethanol (DEPC-H_2O) and then resuspended in 50 ul DEPC-H_2O by vortexing for approx 30 seconds, then heated for 30s at 80 ^oC. This was repeated twice. The RNA was given a final 30s vortex and chilled on ice. RNA was checked by electrophoresis using a fresh gel, if of a good quality, the RNA was dissolved in 4 mls hybridisation buffer (see Table 1) and kept at -20 ^oC for *In situ* hybridisation.

Table 1

HYBRIDISATION BUFFER (50ML)			
Regeant	Volume/ gram	Final concentration	company
formamide	25 ml	50% (V/V)	Sigma, F7508
20 X SSC	12.5 ml	5 X	Invitrogen, AM9763
Torula RNA	50mg	1 mg/ml	Sigma, R6625-25G
heparin	5mg	100µg/ml	Sigma, H3393

50 X Denharts	1ml	1 X	See XX
Tween-20	50ul	0.1%	Promega, H5152
CHAPS	50mg	0.1%	Merck, 44407 4J
0.5 M EDTA	0.5 ml	0.01M	Sigma, E9884
Adjust the final volume to 50ml by adding DEPC-H_2O, then store at -20 ^oC			
50X Denhardt's (100ml)			
Regeant	Volume/ gram	Final concentration	company
Ficoll Type 400	1 mg	1%	Sigma, F8016
Polyvinylpyrrolidone	1 mg	1%	Sigma, PVP360-100G
BSA Fraction V	1 mg	1%	Sigma, A3059
Adjust the final volume to 1L by adding DEPC-H_2O, then store at -20 ^oC			

2.1.5 Site directed mutagenesis

Mutagenesis was performed using the site directed Quick-Change system (Stratagene). Full length Xt PKC lambda cDNA was isolated from the *Xenopus tropicalis* EST database (Gilchrist et al., 2004), the coding sequence clone Tgas015a22 (GenBank AY884235) subcloned into pCS2 (Chalmers et al., 2005). This plasmid was used as a template, CAAX-motif was added with the following primers: CAAX-forward: 5'-CCA CTA CTG ATG TCT GCT TGT GTG CTG TCC TAA TTT CCT CTC GAG G -3'; CAAX-reverse: 5'- GCT CGA GAG GAA ATT AGG ACA GCA CAC AAG CAG ACA TCA GTA GTG G -3'. PCR products were transformed into XL1-Blue supercompetent cells, mini prepped with Wizard Plus SV Miniprep DNA purification

system (Promega, A1330), and sequenced to confirm the change. The resulting plasmid was used to make CAAX-aPKC RNA for injection.

2.2 Fertilisation and injection of *Xenopus* embryos

2.2.1 Injection of adult *Xenopus* to induce ovulation

Priming with PMSG stimulates the development of the ovarian follicle. Adult females were injected in the dorsal lymph sac with 0.5ml 100units/ml PMSG (PMSG is diluted in dH_2O) (Intervet UK). Injection of Human Chorionic Gonadotrophin (HCG) (Intervet UK) stimulates eggs laying and was performed the day before the eggs were required. The primed female was injected with 0.5ml 1000units/ml HCG (HCG is diluted in phosphate buffer) into the dorsal lymph sac. The injected females were placed in a container of water at approx 17/18 ^{o}C. Home Office regulations were observed while carrying out these procedures.

2.2.2 Fertilization and dejellying of *Xenopus* Laevis eggs

Adult males were killed by a Home Office approved schedule I method. The frogs were overdosed with anesthetic by immersing them in 0.03% Benzocaine (in water) (Sigma) approximately 20 min. The heart was destroyed and then the testies removed and placed in 70% L-15 media (Invitrogen) and stored at 4 ^{o}C.

Laying frogs were placed in 1 X Marc's Modified Ringer's solution (MMR) (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM EDTA, 5 mM HEPES, pH 7.8) in a plastic box. Eggs were removed with a 25ml plastic pipette and transferd to

dishes (60x15mm Petri dishes, Fisher, 08-757-360). The 1X MMR was washed off with 0.1xMMR using a cut off polished glass pasteur pipette. The eggs are fertilized with a piece of testis, left for 10 minutes and then flooded with 0.1x MMR.

If performing injections, the jelly coat of the embryos was removed with cysteine solution at least 40 mins post fertilization. The embryos are treated with 2.5% cysteine (2.5% cysteine, pH to 7.8-8.1; L-Cysteine hydrochloride monohydrate, Sigma, C7880), then washed with 0.1xMMR 3-4 times, and transferred to a clean petri dish.

2.2.3 Injection of *Xenopus* laevis embryos

Injection needles were prepared from glass capillaries (Drummond Scientific Co., Broomall, PA, USA; 3.5" Drummond-3-000-203-G / X, 100 needles) using a needle puller (Flaming/Brown micropipette Puller, P-97, SUTTER Instrument CO.). Embryos were placed in agarose coated dish with injection buffer (1% Ficoll and 0.5X MMR in autoclaved dH_2O). 10 nanoliters RNA solution was injected by using a Nanoject injector (Drummond Scientific Co., Broomall, PA, USA). After injection embryos were kept in the injection buffer for at least an hour to heal, then placed in 0.1X MMR and cultured until required.

2.3 *In Situ* hybridization

2.3.1 X-Gal staining

β-gal RNA was often injected with the experimental RNA as a tracer. At the desired stage embryos were fixed for 1 hour in MEMFA (see Table 2). Embryos were washed

for 5 minutes at room temperature in 0.1M Sodium phosphate buffer (pH 6.2). Sodium phosphate buffer was replaced with fresh β-gal solution (see Table 3) and incubated at room temperature until the staining was well developed. The reaction was stopped by rinsing twice with 0.1M sodium phosphate buffer. The embryos were then dehydrated as follows: 2 minutes 50% ethanol, 2 minutes 70% ethanol and twice 2 minutes 100% ethanol. Then stored in ethanol at –20^{o}C.

Table 2

10XMEM SALT (500ML)			
Regeant	Volume/ gram	Final concentration	company
MOPS	104.5 g	1 M	Sigma, M9381
EGTA	3.8 g	20 mM	Sigma, E3889
MgSO4	1.24 g	10mM	
Dissolved in DEPC-H_2O, adjusted PH to 7.4, filter sterilized and stored at 4 ^{o}C			
MEMFA (50ml)			
10 x MEM Salt	5 ml	1 x	
37% formaldehyde	5 ml	1/10 V	Sigma, F8775
Adjust volume with H_2O to 50ml			

Table 3

0.1M SODIUM PHOSPHATE BUFFER (1L)			
Regeant	Volume/ gram	Final concentration	company
0.5 M Na_2HPO_4	35.6ml		
1 M NaH_2PO_4	82.2ml		
Adjust PH to 6.2 and volume with H_2O to 1 L, then autoclave			

15% X-gal (500ul) (Make fresh)			
X-Gal	0.075 g	15 %	Apollo Scientific
Dissolved in 500ul DMF (N,N-Dimethylformamide) (Sigma, D4551)			
β-gal solution (50ml) (Make fresh)			
Potassium Ferricyanide	0.165 g	10 mM	Sigma, 393517
Potassium Ferrocyanide	0.211 g	10 mM	Sigma, P3289
15% X-Gal	500ul		
Dissolved in 0.1M sodium phosphate buffer, adjusted volume to 50ml			

2.3.2 Whole Mount *In Situ* Hybridization

The protocol for *In situ* hybridisation was based on a previously described method (Harland, 1991). Embryos were fixed at the required stage in MEMFA for 1-2 hours at room temp and dehydrated in ethanol and stored at -20 ^{o}C until required. Embryos were rehydrated by 5 minutes washes in 100% ethanol, 75% ethanol (25% H_2O), 50% ethanol (50% H_2O), 25% ethanol (75% PTw), then washed 3 x 5 minutes in 100% PTw (0.1%Tween-20 in 1xPBS, Tween-20 from Promega, H5152). Incubated for 5 minutes at room temperature in 5ml Proteinase K (Sigma, P6556, dissolved in dH_2O with the concentration of 25mg/ml) (10µl 25mg/ml Proteinase K in 50ml PTw). Embryos were then rinsed twice for 5 minutes in 5ml of 0.1M triethanolamine (Sigma, T1377) (dissolved in dH_2O, and adjusted to pH 7.8). 12.5µl acetic anhydride (ScholAR Chemistry) was added to the last 5ml 0.1M triethanolamine, and rocked for 5 minutes. Then another 12.5µl acetic anhydride was added and rocked for 5 minutes. Embryos were washed 2 x 5 minutes in 5ml PTw and then fixed for 20 minutes in 4% formaldehyde (Sigma, F8775) (4% formaldehyde in PTw). After that, washed 5 x 5

minutes in PTw. Embryos were transferred into the 2ml screw cap plastic vial (Wheaton, 03-341-18) with 500µl hybridization buffer and prehybridized for 4-6 hours at 60°C in water bath. The hybridization buffer was replaced with 0.5ml of probe solution (hybridization buffer with 1µg/ml probe) and hybridized overnight at 60°C.

The probe was removed next morning and replaced with hybridization buffer. Embryos were washed at 60°C for 10 minutes then washed 3 x 20 minutes at 60°C in 2x SSC. Treated for 30 minutes at 37^{o}C with 2x SSC with 20 µg/ml RNase A (Sigma, R5000; dissolved in dH_2O) and 10 µg/ml RNAse T1 (Sigma, R1003-100KU), then washed in 2xSSC at room temperature and twice for 30 minutes at 60^{o}C in 0.2xSSC. Embryos were washed twice in 1xMAB (2x MAB stock: 23.2g Maleic acid, 15.6g NaOH, 17.5g NaCL in 1L H_2O, PH 7.5) for 10-15 minutes, then pre-blocked from 15 minutes to 1 hour in 1ml 1xMAB + 2% BMB (Boehringer Manheim Blocking Reagent, Roche, 1 096 176; dissolved in 1xMAB to make 10% stock, autoclaved and aliquoted, then stored in -20^{o}C). Blocked for 1hour in 1 ml 1xMAB + 2% BMB + 20% heat treated lamb serum (HTLS, Sigma, S2263) (2ml 10% BMB and 2ml HTLS to 6ml 1xMAB). Blocking buffer was replaced with MAB + 2% BMB Blocking Reagent + anti-Digoxigenin-AP (1/2000 dilution of the anti-digoxygenin alkaline phosphatase antibody (Anti-Digoxigenin-AP, Fab fragments from sheep, Roche, 11093274910) for 4 hours at room temperature. Followed with 2 washes for 10-20 minutes each in 1xMAB, embryos were then washed overnight in 1x MAB at 4^{o}C.

Embryos were washed for 2x 30 minutes in 1x MAB at room temperature, then twice for 5 minutes with 2ml fresh made Alkaline Phosphatase (AP) buffer (100mM Tris,

PH 9.5, 50mM $MgCl_2$, 100mM NaCl and 0.1% Tween-20). NBT/BCIP substrate diluted in AP buffer, 0.338 µg/ µl NBT (Sigma, N6639) and 0.175 µg/ µl of BCIP (Roche, 1 383 221) was added and once the colour developed, the embryos were rinsed twice in PTW for 15 minutes to stop the reaction. Embryos were placed in MEMFA and replaced with new MEMFA.

2.3.3 Embedding in Gelatin Albumen Mixture

To section embryos after *in situ* hybridisation, the MEMFA fixed embryos were soaked in a small volume of EMB (see Table 4) for 30 mins. 140µl of 25% gluteraldehyde (Grade I, Sigma, G5882) was added to 2 mls of EMB in a weigh boat, mixed rapidly with the pipette tip, then poured into a watch glass and left for a couple of minutes. The embryo was placed on the set EMB in the watch glass. Another batch of EMB and 140µl of 25% gluteraldehyde (Grade I, Sigma, G5882) was poured over the top of the embryo. The block was left to solidify and trimmed. The block was attached to the vibratome (Leica, VT1000S). Sectioning stage with superglue and placed in the vibratome reservoir filled with 1xPBS. Sections were cut at 30 microns minimum thickness. The sections were transferred onto slides, mounted in 90% glycerol and then sealed with nail varnish. The images were captured with Nikon sight DS-U1 camera (Nikon) together with NIS Elements F software (Nikon, JP).

Table 4

EMB		
Regeant	Volume/ gram	company
Gelatin, type A	0.815 g	Sigma, G1890
sucrose	33.3 g	Sigma, S9378

Albumin, Bovine	50 g	Sigma, A3912
Dissolved in 167ml 1x PBS, then aliquoted and stored in -20^{o}C		

2.4 Immunohistochemistry

Cryosections were prepared based on the previously described method (Fagotto and Gumbiner, 1994). Embryos were fixed at the required stage with MEMFA for 1 hour, then rinsed in methanol and stored at -20°C. The embryos were rinsed with wash solution (0.1M Tris and 0.1 M NaCl, pH 7.4), and embedded in 15% cold water fish gelatine (15% cold water fish gelatine, Sigma, G1890; and 15% sucrose, Sigma, S9378; dissolved in water) at room temperature for 24 hours. Then embryos were further embedded in 25% cold water fish gelatine (25% cold water fish gelatine, Sigma, G1890; and 15% sucrose Sigma, S9378; dissolved in water) at room temperature for 24 hours. Embryos were placed in a square mould (Lamb) full of 20% cold water fish gelatine (20% cold water fish gelatine, Sigma, G1890; and 15% sucrose Sigma, S9378; dissolved in water) and frozen on dry ice for at least 20 minutes. 10um sections were cut at -27°C by using a cryostat (Microm, Zeiss) and collected on precoated glass slides (Fisher ScientificCo.,Springfield, NY, USA), stored at -80°C.

Sections were then washed in acetone, PBS and blocked in PBS+1% BSA (Sigma, A 3059) +5% HTLS serum (Sigma, S2263) for 30 minutes incubated with primary antibody for 2 hours, washed three times in PBS, blocked in PBS+1% BSA (Sigma, A 3059) +5% HTLS serum (Sigma, S2263), incubated with the secondary antibody for 1 hour, washed three times in PBS and mounted in Vectashield (Vector Laboratories).

Sections were examined and images were obtained on a Zeiss LSM510 (Zeiss) laser scanning confocal microscope.

The following antibody combinations were used: rabbit anti-PKC-ζ (C-20) (1:150) (Santa Cruz, sc-216), mouse anti-ZO-1 (1/25) (Invitrogen, 339100), rabbit anti-Phospho-Smad1/Smad5/Smad8 (1/150) (Cell Signaling Technology, 9511), mouse anti- Alexa 586 (1:500) (Molecular Probes, A11004) and rabbit anti-Alexa 586 (1:500) (Molecular Probes, A11011); DAPI (Molecular Probes) was used as a nuclear stain added with the secondary antibody at final concentration of 1/1000.

2.5 Cell dissociation

Animal caps were cut in 0.5 X MMR at stage 9-10. The superficial layers and deep layers were dissociated separately by rinsing in calcium and magnesium free media (CMFM) (88 mM NaCl, 1 mM KCI, 2.4 mM $NaHCO_3$, and 7.5mM Tris, pH 7.6). Gently swirling animal caps in CMFM media in agarose (Agarose MP, Roche, 11388983001) coated dish. Rolling epithelial layers and dispersed deep cells were collected separately for RT-PCR.

For experiments in which BMP4 protein was used, dispersed deep cells were cultured in CMFM media with 0.5mg/ml BSA (Sigma, A 3059) + 100ng/ml purified human BMP4 protein (PeproTech). Cells were cultured for 5 hours to stage 12 and 17 hours to stage 18. Batches of cells were cultured with CMFM and BSA (Sigma, A 3059) as a control.

Table 5 Plasmids used in the project

NAME	STOCK NO.	LINEARIZE	TRANSCRIPTION	USAGE
GFP	39	Not I (NEB, R0189)	Sp6 (Promega, P1081)	overexpression
BMP4	16	XbaI (Promega, R6185)	Sp6 (Promega, P1081)	overexpression
Grhl3	247	Not I (NEB, R0189)	Sp6 (Promega, P1081)	overexpression
Lac Z	15	Not I (NEB, R0189)	Sp6 (Promega, P1081)	overexpression
Noggin	12	EcoRI (NEB, R0101)	Sp6 (Promega, P1081)	overexpression
Active ALK2	XX	NotI (NEB, R0189)	Sp6 (Promega, P1081)	overexpression
GFP-Lgl	220	Not I (NEB, R0189)	Sp6 (Promega, P1081)	overexpression
C-aPKC	270	Not I (NEB, R0189)	Sp6 (Promega, P1081)	overexpression
CAAX-aPKC	310	Not I (NEB, R0189)	Sp6 (Promega, P1081)	overexpression
Claudin 4	256	BamH I (NEB, R0136)	T7 (Promega, P2075)	*In situ* probe
Keratin I	75	Not I (NEB, R0189)	T7 (Promega, P2075)	*In situ* probe
Hyaluronan synthesis I	253	EcoRI (NEB, R0101)	T7 (Promega, P2075)	*In situ* probe

Pyrothymosin alpha	255	EcoR I (NEB, R0101)	T7 (Promega, P2075)	*In situ* probe
Keratin II	273	BamH I (NEB, R0136)	T7 (Promega, P2075)	*In situ* probe
Sox3	23	BamH I (NEB, R0136)	T7 (Promega, P2075)	*In situ* probe
N-tubulin	9	BamH I (NEB, R0136)	T3 (Promega, P4024)	*In situ* probe
Occludin	334	BamH I (NEB, R0136)	T7 (Promega, P2075)	*In situ* probe
Cingulin	333	SpeI (Promaga, R6591)	T7 (Promega, P2075)	*In situ* probe
Xt PKC lambda	266	—	—	site directed mutagenesis

2.6 RT-PCR

RT-PCR was presented according to a previously described method (Richardson et al., 1995; Hudson et al., 1997). RNA was isolated from groups of 20 isolated layers and extracted with 150 ml XTB (300 mM NaCl, 20 mM Tris (pH 7.5), 1 mM EDTA, 1% SDS, 0.6 mg/ml Proteinase K) with 10 mg of glycogen as a carrier. An equal volume of phenol was added followed by vigorous vortexing. The tube was left on ice for 10 minutes with brief vortexing, then spun in a microcentrifuge at 12,000rpm for 5 minutes. RNA was resuspended in 60ul DNase I buffer (20 mM Tris-Cl, pH 8.3; 50 mM NaCl, 2.5 mM $MgCl_2$), containing 20 units DNase I (Roche, 04716728001) and 10 units of RNase inhibitor (Roche, 11119915001) and incubated for 30 minutes at

37°C. The sample was re-extracted with phenol, then chloroform/isoamyl alcohol (24:l) and precipitated with 2 volumes of ethanol at -20°C for 1 hour, resuspended in 0.5 ml XTB and incubated at 37°C for 15 minutes, followed by a second extraction and precipitation as described above. The dried pellet was resuspended in 20ul DEPC-H_2O and stored at -80°C.

To make cDNA RNA was denatured at 75°C for 5 minutes, and then cooled on ice. Reverse transcription reactions (20ul) contained 2 mM random hexamers (Invitrogen, N8080127), 4ul 5 X AMV buffer (Roche, 10109118001), 20mM dNTPs (Promega, U1330), 25units RNase inhibitor (Roche, 11119915001) and 40 units Reverse Transcriptase, AMV (Roche, 10109118001). After incubation for 1 hour at 42°C, reactions were terminated by heating at 95°C for 5 minutes.

PCR reactions were carried out in a 20ul volume: 1ul of reverse transcription reaction in 13 ul PCR buffer with the addition of 1.5 mM $MgCl_2$, 200 mM dNTPs, 1 mM each primer (ordered from MNG), and 0.5 units of Taq DNA polymerase (except primers, all PCR reagents came from Taq DNA Polymerase (1 U/µl), dNTPack, Roche, 04738225001). PCR programs were presented as follow: initial denaturation at 94°C for 3 minutes; cycles begin with denaturation at 94°C for 30 seconds, then annealing at primer dependent temperature for 45 seconds, elongation at 72°C for 45 seconds, the programs ran 33-35 cycles; final elongation at 72°C for 5 minutes. The products were visualized on a 2% agarose gel. Loading controls were run with each marker to ensure that the reaction was in the linear range. Details of every primer were summarised in Table 6.

Table 6 *Xenopus* RT-PCR primers

GENE	PRIMERS SEQUENCE	ANNEALING TEMPERATURE
Msx1	5'-act ggt gtg aag ccg tcc ct -3' 5'-ttc tct cgg gac tct cag gc-3'	55◦C
Grhl1	5'-tga cca ccg cct tca gtg ct-3' 5'-cct tgg ctg ccc tga cat tg-3'	56◦C
Grhl3	5'-cga tgg aag cac tgg cac tc-3' 5'-cca cat ctt tga aga ttg g-3'	53◦C
CtBP	5'-atg agc aca acc atc acc-3' 5'-cag aag gta tga gag gat gc-3'	55◦C
AP-2	5'-cgg gta tgt gtg cga aac ag-3' 5'-ggc ggg aga cca ata gag aa-3'	56◦C
Vent-2	5'-ctc ata ctc cag agg aat gg-3' 5'-tgg atg cat ggt ata ggg-3'	55◦C
Cdx2	5'-aag gtt cca agc tca agg-3' 5'-ccc att cat ctt cgt tgc-3'	55◦C
Vestigial –like 4	5'-tcg gca aga act aca agg-3' 5'-aca ctc acg cca ata agc-3'	55◦C
ODC	5'-cag cta gct gtg gtg tgg-3' 5'-caa cat gga aac tca cac c-3'	55◦C

2.7 mESCs techniques

2.7.1 ESC cell lines (Sox1-GFP and R63)

R63 is an ESC line established from E14tg2a cells clone 63, which stably express a

transfected tetracycline-regulated transactivator construct pCAG20-1 (Era and Witte, 2000).

46C (Sox1$^{+/gfp\text{-ires-}pac\text{-}pA}$) cells were established from parental E14tg2a cells (Nichols et al., 1998; Niwa et al., 2002). The *Sox1* gene open reading frame was replaced by GFP coding sequence and an internal ribosome entry site (IRES) linked to a puromycin resistance gene. By using the above protocol, Ying and his colleagues developed Sox1-GFP knock-in (46C) ES cells which express GFP driven by the Sox1 promoter (Ying et al., 2003a).

2.7.2 mESCs culture

Murine ES cell lines were routinely cultured according to previously described method (Paling et al., 2004). mESCs were cultured on tissue culture plates (Nunc) coated with 0.1% (v/v) porcine gelatin (Sigma, G2500) in knock-out Dulbecco's modified Eagle's medium (Invitrogen, 10829-018) in the presence of 15% (v/v) knock-out serum replacement (Invitrogen, 10828028), 0.1 mm 2-mercaptoethanol (Bio-Rad), 1 mm sodium pyruvate (Fisher Scientific 11360), 2 mm glutamine (Invitrogen 25030-024), 0.1 mm non-essential amino acids (Invitrogen 11140-050), and 1000 units/ml murine LIF (Chemicon, ESG1106). Cells were trypsinized and replated or re-fed every second day with the cell density 2-5 x 10^5 per dish. Cell density was counted and calculated with the aid of hemocytometer. Cells were maintained at 37°C, 5% CO_2.

2.7.3 N2B27 media

Standard culture media for mESCs contains a variety of growth factors, which are found in serum containing and serum-replacement media. Although those kinds of media can support mESCs' growth well, they are not suitable to analysis the role of a single growth factor in mESCs self-renewal or differentiation due to the complicated and unknown ingredients. N2B27 media was originally used for a rat neuroblastoma cell culture (Brewer, 1993). However, Ying and co-workers refined the media for mESCs culture (Ying et al., 2003a, Ying et al., 2003b). N2B27 media is a serum free chemically defined media with known components. It makes it possible to investigate the contribution of single growth factors in mESCs self-renewal/ differentiation. N2B27 is a 1:1 mixture of DMEM/F12 (Invitrogen, 21331-020) supplemented with modified N2 (Invitrogen, 17502048) and Neurobasal medium (Invitrogen, 21103-049) supplemented with B27 (Invitrogen, 17504-044), 2mM glutamine (Invitrogen 25030-024), 50μM Bovine Serum Albumin (BSA) fraction V (Sigma, A3059), 0.0125% (v/v) Monothioglycerol (MTG) (Sigma, M6145).

2.7.4 Differentiation in N2B27 media using Monolayer cell culture

Monoculture differentiation was based on the method previously described (Ying et al., 2003a). Undifferentiated mESCs were dissociated and plated onto a 0.1% gelatin-coated 0.4μm pore polyester membrane insert of a 6.5mm Transwell dish (CORNING, #3470) or tissue culture plates (NUNC Tissue Culture dish 92x17mm, Fisher Scientific, TKT-110-070A) at a density of $1.68 \times 10^4/cm^2$ in N2B27 medium plus 1000 units/ml LIF (Chemicon, ESG1106) and 10ng/ml BMP4 (PeproTech), or in N2B27 media with 10ng/ml BMP4, or in N2B27 media without BMP4 and LIF. Cells

were maintained at 37°C, 5% CO_2.

2.7.5 Immuno-fluorescence staining

Cells were grown on a 0.4μm pore polyester membrane insert (CORNING, #3470) 4-5 days prior to fixation and processed for immunofluorescence microscopy as follows. Cells on a membrane insert were rinsed with PBS and fixed with 4% (w/v) paraformaldehyde (Sigma, P6148) for 1 hour and were permeabilized using methanol at -20◦C for 5 minutes. They were then washed 3 times in PBS, blocked in PBS containing 10% FCS (Invitrogen, 26400-036) for 30 minutes and then washed twice in PBS containing 2% FCS (Invitrogen, 26400-036). Primary antibodies were diluted in PBS containing 2% FCS (Invitrogen, 26400-036) and incubated with the cells for 2 hours at room temperature. Appropriate fluorophore-conjugated secondary antibodies diluted in PBS containing 2% FCS (Invitrogen, 26400-036) were incubated with the cells for 1 hour at room temperature. Cells were washed five times for each 5minutes in PBS containing 2% FCS (Invitrogen, 26400-036) following all antibody incubations. Polyester membrane were cut from insert and mounted in Vectashield (Vector Laboratories). Cells were examined and images were obtained on a Zeiss LSM510 laser scanning confocal microscope.

The following antibody combinations were used: mouse anti-ZO-1 (1/25) (Invitrogen, 339100), mouse anti-E-Cadherin (1/25) (Invitrogen, 131700), mouse anti-Cdx2 (1/100) (Santa Cruz, sc-166830), rabbit anti- HNF4a (1:100) (Santa Cruz, sc-8987), rabbit anti-Oct3/4 (1:200) (Santa Cruz, sc-133866), mouse anti-Troma1 (Developmental Hybridoma Bank). The following secondary antibodies were used mouse anti-Alexa 586 (1:500) (Molecular Probes, A11004), rabbit anti-Alexa 586

(1:500) (Molecular Probes, A11011). DAPI (Molecular Probes) was used as a nuclear stain added with the secondary antibody at final concentration of 1:1000.

2.8 Cell counting

Quantification of the cells surrounding the colonies was carried out to establish the percentage of cells that were positive for each marker used in the immunofluorescence. It was carried out as follows. At least four colonies with surrounding differentiated cells were randomly selected from each filter with the aid of the DAPI stained nuclei. Using the Zeiss LSM510 laser scanning confocal microscope, the number of positive/negative cells and the total number of cells was then counted. This gave an average of more than 200 cells per experiment. The mean of at least three independent experiments, +/- the standard deviation, was then calculated and shown graphically with GraphPad Prism 3.0 software (GraphPad Software, San Diego, CA, USA).

2.9 RT-PCR (ES cell)

Cells cultured for 5 days were collected from N2B27 media only, N2B27 media with both BMP4 and LIF, and N2B27 media with BMP4 dishes. Total RNA extraction was carried out according to manufacturers instructions using Trizol (Invitrogen).

For RT-PCR analyses, 1 µg total RNA was reverse transcribed using Oligo(dT)$_{12-18}$ Primer primers (Invitrogen, 18418-012) and SuperScript™ II Reverse Transcriptase (Invitrogen, 18064-014). PCR was performed on 1/20 of the final cDNA volume.

PCR reactions were carried out in a 20ul volume: 1ul of reverse transcription reaction in 13 ul PCR buffer with the addition of 1.5 mM $MgCl_2$, 200 mM dNTPs, 1 mM each primer (ordered from MNG), and 0.5 units of Taq DNA polymerase (except primers, all PCR reagents came from Taq DNA Polymerase (1 U/μl), dNTPack, Roche, 04738225001). PCR programs were presented as follow: initial denaturation at 94°C for 3 minutes; cycles begin with denaturation at 94°C for 30 seconds, then annealing at primer dependent temperature for 45 seconds, elongation at 72°C for 45 seconds, the programs ran 33-35 cycles; final elongation 72°C for 5 minutes. The products were visualized on a 2% agarose gel. Loading controls were used to ensure that PCR was in the linear range. Details of every primer were summarised in Table 7.

Table 7 ES cell RT-PCR primers

genes	Primer sequence	types	annealing temperature
BETA-actin	5'-taggcaccagggtgtgatgg-3' 5'-catggctggggtgttgaagg-3'	housekeeping gene	60◦C
Oct3/4	5'-cacgagtggaaagcaactca-3' 5'-agatggtggtctggctgaac-3'	pluripotency	58◦C
Nanog	5'-cacccacccatgctagtctt-3' 5'-accctcaaactcctggtcct-3'	pluripotency	58◦C
HNF4α	5'-acaggagagggtcagaagca-3' 5'-gatgtttgcacaaccacagg-3'	primitive endoderm	58◦C
Gata4	5'-atctctgcatgtcccatacc-3' 5'-tctgacttaagagggcttgg-3'	primitive endoderm	53◦C
AP2-alpha	5'-tcatgggactaactcatcgc-3' 5'-ggaagttcaagtgggtggtt-3'	early surface ectoderm	60◦C
p63	5'-gcatggattgtatccgcatg-3'	early surface ectoderm	58◦C

	5'-gccccaggttcgtgtactgt-3'		
Cdx2	5'-aggctgagccatgaggagta-3' 5'-cgaggtccataattccactca-3'	trophectoderm	58◦C
Dlx3	5'-gccttaggggtaaggctgtc-3' 5'-gacctgcttctcttggttgc-3'	trophectoderm	55◦C
Eomes	5'-tgatcatcaccaaacagggc-3' 5'-actgtgtctctgagaaggtg-3'	trophectoderm	60◦C
Fgfr2	5'-cactgagcagagaggctgtg-3' 5'-ggcggctgtcactatcaga-3'	trophectoderm	63◦C
Gata3	5'-gggctacggtgcagaggtat-3' 5'-tggatggacgtcttggagaa-3'	trophectoderm	58◦C
Psx1	5'-gaattggtttcggatgagga-3' 5'-gtggctcagaagaagccatc-3'	trophectoderm	55◦C
Mash2	5'-cgggatctgcactcgaggat-3' 5'-ggtgggaagtggacgtttgc-3'	ecto-placental cone	60◦C
Psg19	5'- gacgctttcaactctgtcca-3' 5'-cacggccactgatgatagac-3'	ecto-placental cone	60◦C
Tpbp	5'-aagttaggcaacgagcgaaa-3' 5'-agtgcaggatcccacttgtc-3'	ecto-placental cone	55◦C
PL2	5'-tccttctctggggcactcctgtt-3' 5'-ccatgaaggcttttgaagcaagatca-3'	trophoblast giant cell	60◦C
Grhl1	5'-gaagcactactccaatgagg-3' 5'-accactcttatggacacagg-3'	transcription factor	55◦C
Grhl3	5'-acaacctcttctgtgtcagc-3' 5'-ggcaagggtatttgttcc-3'	transcription factor	53◦C
Msx1	5'-cctcaagctgccagaagatg-3' 5'-agctgagctgtggtgaaagg-3'	transcription factor	58◦C
Msx2	5'-tcttcgcttgagagttgc-3'	transcription factor	56◦C

	5'-tggacaggtactgtttctgg-3'		

3 Chapter III aPKC and Notch

3.1 Introduction

To choose candidates that might promote superficial cells differentiation, several aspects could be considered, this chapter will focus on two candidates, which act as cell fate determinants during asymmetric cell division. These are aPKC and Notch.

3.1.1 PAR-aPKC system controls cell polarity and cell fate

aPKC has a well established role in regulating cell polarity (Chapter I). A Recent report revealed that aPKC also acts as a cell fate determinant in neuroblasts (Lee et al., 2006). Wild type neuroblasts undergo asymmetric cell division, generating one neuroblast by self-renewal and one GMC which differentiates (Lee et al., 2006). By investigating the number of neuroblasts and GMCs, in both Lgl mutants and Lgl aPKC double mutants, the requirement of aPKC for the neuroblast self-renewal was demonstrated (Lee et al., 2006). aPKC was also shown to be sufficient to promote neuroblast cell fate. By comparing three different aPKC constructs, membrane-target aPKC, membrane-target kinase-dead aPKC and constitutively active aPKC, membrane-target aPKC was demonstrated to be the best at promoting neuroblast cell fate. Their overexpression revealed two essential elements for aPKC function, membrane targeting and the kinase activity, which are important for neuroblast specification (Lee et al., 2006).

In *Xenopus* blastomeres, the oriented cell divisions generate cells with different molecular components. aPKC localizes to the apical membrane throughout the early cleavage stages and the membrane localised aPKC is inherited only by superficial cells (Chalmers et al., 2003). This is similar to the situation in *Drosophila* neuroblasts and suggests that aPKC might work as an epithelial cell fate determinant during formation of *Xenopus* superficial and deep cells.

3.1.2 Notch can regulate cell fate decisions after asymmetric cell divisions

Notch was first discovered as a neurogenic fate switching factor (reviewed by Greenwald, 1998). In *Drosophila*, the Notch protein was characterized as a 300-KD single pass transmembrane receptor (Wharton et al, 1985). After that, Notch proteins were found in many other species (reviewed by Greenwald, 1998).

Notch signaling is highly conserved in multicellular organisms (Greenwald, 1998). The Notch cascade consists of the Notch receptor and Notch ligands (reviewed by Gridley, 1997). The Notch ligands are members of the DSL (Delta，Serrate/Jagged，Lag-2) family of proteins (Wharton et al, 1985). Their N-terminal DSL domain is essential for interactions with Notch receptors (reviewed by Artavanis et al, 1999). The interaction between Notch and Notch ligands from neighboring cells will activate Notch and cause cleavage of the Notch transmembrane domain and release of its intra-cellular domain (Notch-ICD) to the nucleus, where it regulates target genes transcription (Kidd et al., 1998).

Notch is another molecule involved in regulating *Drosophila* neuroblast self-renewal. After asymmetric cell division, two daughter cells are produced with an asymmetric inheritance of Numb (Chapman et al., 2006). In one daughter cell, the absence of Numb allows Notch activation which blocks differentiation. The inheritance of Numb in the other daughter cell acts to inhibit Notch signaling, allowing differentiation to occur (Chapman et al., 2006). The negative regulation of Numb and Numblike by Notch is then essential for stable cell fate specification (Fan et al. 2006).

Notch has also been shown to participate in vertebrate neurogenesis (reviewed by Artavanis-Tsakonas et al. 1999). In *Xenopus*, Notch/Delta mediates lateral inhibition during primary neurogenesis, which takes place in the deep layer of the neuroectoderm (Bellefroid et al. 1998). Compared to deep layer precursors, superficial layer precursors are refractory to the neuronal-promoting signals (Chalmers et al., 2002). ESR6e is a bHLH protein of the Enhancer-of spilit/hairy/HES family which is expressed in the superficial layer (Deblandre et al., 1999) and able to act as an inhibitor of primary neurogenesis (Chalmers et al., 2002). ESR6e expression is activated by ectopic Notch (Deblandre et al., 1999). This suggests that Notch may be involved in activating ESR6e in the superficial layer of *Xenopus* embryos. Based on Notch's role in asymmetric cell division and its possible regulation of ESR6e, it could be that the Notch pathway promotes superficial cell fate.

The aim of this chapter is to test candidates that could act to promote superficial

specification. These candidates are aPKC and Notch. The strategy is to overexpress the candidate RNA into *Xenopus* deep cells and confirm the candidate protein is active. The expression of superficial markers in the deep cells was then examined to test the ability of the candidate to switch deep cell fate to superficial cell fate.

3.2 Results

3.2.1 Injected *aPKC* is active in the deep cells

In order to promote superficial cell fate, it is necessary to use the most active form of aPKC in the overexpression system. Compared with wild type aPKC, C terminal aPKC (C-aPKC) is persistently active as it lacks autoinhibition from the regulatory domain (Hernandez et al., 2003; Lee et al., 2006). This makes the C-aPKC construct more potent than wild type aPKC in promoting apicalisation of superficial cells (A Chalmers, personal communication), as well as active in promoting neuroblast self-renewal in *Drosophila* (Lee et al., 2006). The membrane targeted form of aPKC works even better than C-aPKC at promoting neuroblast cell fate (Lee et al., 2006). This construct has a CAAX-motif which causes the protein to associate with membranes.

To confirm the CAAX-motif was working correctly, the localization of overexpressed CAAX-aPKC was examined. Two groups of RNA were injected into *Xenopus* embryos, *C-aPKC* and *CAAX-aPKC*. Embryos at stage 11 were sectioned and IF stainned with rabbit anti-PKC-ζ (C-20) antibody, it was revealed that C-aPKC

localized mainly in nucleus and in regions adjacent to the plasma membrane (Fig 3.1 A-C). There was also cytoplasmic staining, particularly around the nucleus. This was not even through out the cytoplasm due to the large amount of yolk in these cells. The CAAX-aPKC associated with membrane structures, such as the plasma membrane and nuclear envelope and there was no strong CAAX-aPKC staining found in the nucleus (Fig. 3.1 D-F). This demonstrated that the CAAX domain was causing aPKC to associate with membranes.

To check if the overexpressed protein was active in deep cells, the inhibition of Lgl by aPKC was used. Three groups of RNA were injected into *Xenopus* embryos at the 2-cell-stage, *Lgl-GFP* (1ng/embryo), *Lgl-GFP* and *C-aPKC* with the proportion of 1:1 (0.5ng *Lgl-GFP* + 0.5ng *C-aPKC* / embryo) and *Lgl-GFP* and *CAAX-aPKC* with the proportion of 1:1 (0.5ng *Lgl-GFP* + 0.5ng *CAAX-aPKC* / embryo). Embryos at stage 11 were sectioned and IF stainned with rabbit anti-PKC-ζ (C-20) antibody, there were significant differences between the three groups in the localization of Lgl (Fig.3.2). The Lgl-GFP control localized to the basal domain (Fig 3.2 B, D). C-aPKC changed the Lgl-GFP localisation, Lgl-GFP displayed more cytoplasmic localisation (Fig 3.2 F, H). Similar results were found in the *CAAX-aPKC* injected embryos, after injection with *CAAX-aPKC* the Lgl-GFP displayed more cytoplasmic localisation (Fig 3.2 J, L) than control Lgl-GFP (Fig 3.2 B). The results indicated that *aPKC* injection inhibited Lgl-GFP localization. Thus, the *C-aPKC* or *CAAX-aPKC* overexpressed in *Xenopus* embryos was confirmed to be active.

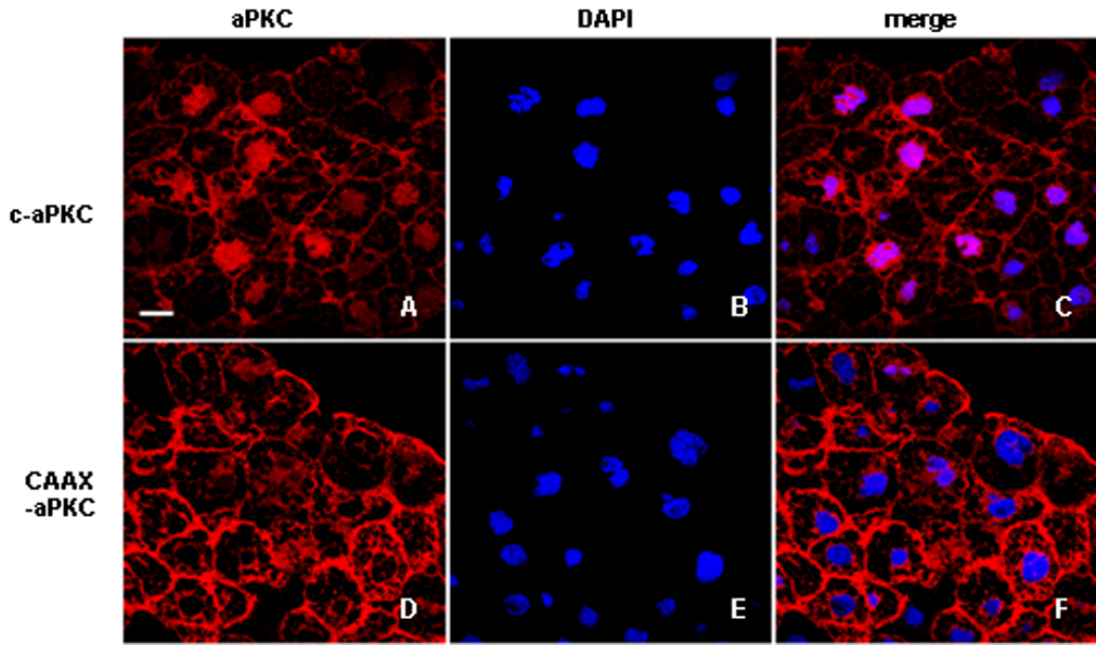


Fig 3.1. Localization of c-aPKC and CAAX-aPKC. (A-C) Localisation of C-aPKC. (D-F) Localisation of CAAX-aPKC. The animal cap region of a stage 11 embryo is shown for each construct. aPKC (red), DNA (Blue). Scale bar equals 10 μm. Based on at least 3 independent experiments.

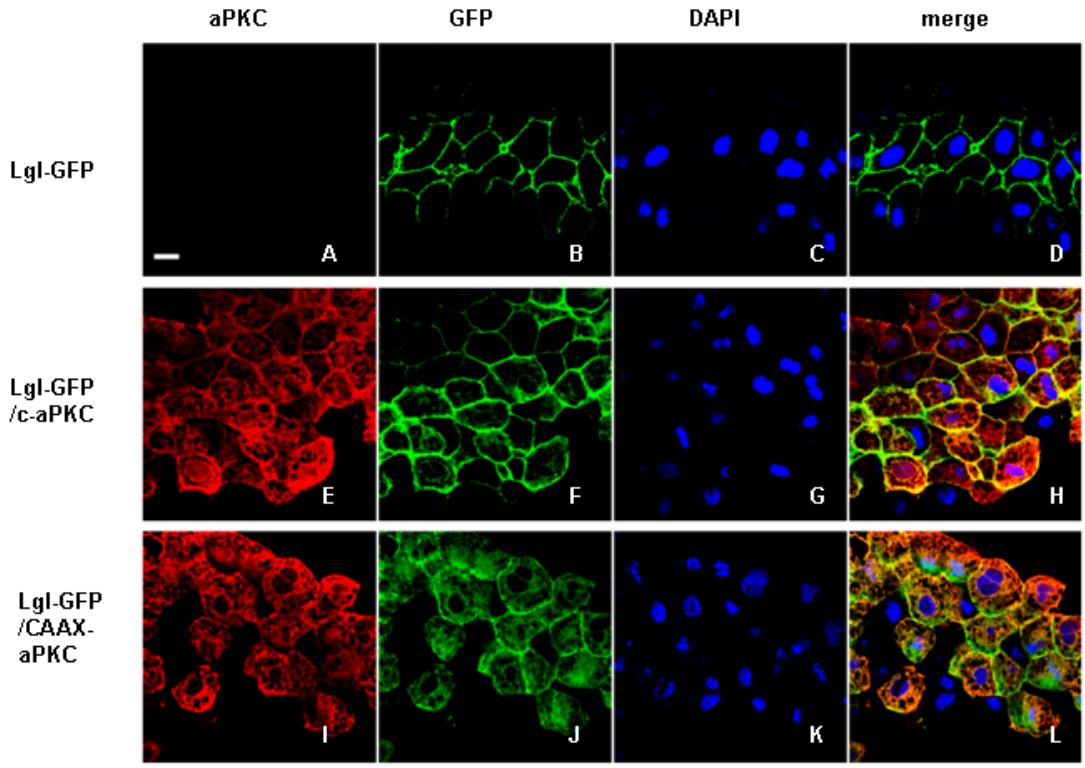


Fig 3.2. C-aPKC and CAAX-aPKC expression inhibited the membrane localization of LGL. (A-D) Localisation of the Lgl-GFP in control embryos. (E-H) Localisation of the Lgl-GFP after injection with *C-aPKC*. (I-L) Localisation of the Lgl-GFP after injection with *CAAX-aPKC*. The animal cap region of a stage 11 embryo is shown, aPKC (red), GFP (Green), DNA (Blue). Scale bar equals 10 μm. Based on at least 3 independent experiments.

3.2.2 Overexpressed *Notch-ICD* is active in the deep cells

Notch signaling is widely used to determine cell fate and regulate pattern formation. Active Notch pathway can be achieved by overexpressing the intracellular domain of Notch (Notch-ICD), which constitutively translocates to the nuclei (Struhl and Adachi, 1998). It is known that Notch-ICD blocks the formation of ciliated cells, which can be visulised by α-Tubulin expression (Deblandre et al., 1999). This provides a strategy to confirm the activity of *Notch-ICD* by investigating its effect on *α-Tubulin*. Two groups of RNA were injected into *Xenopus* embryos, *GFP* (1ng/embryo) and *Notch-ICD* (1ng/embryo). *Lac Z* RNA was injected as a lineage label with the experimental RNA. *α-Tubulin* expression was assayed by *in situ* hybridization at stage 13 (Fig 3.3). *GFP* control displayed the normal expression of *α-Tubulin* (Fig 3.3 A, A', A''). However, *α-Tubulin* expression was strongly inhibited by *Notch-ICD* overexpression (Fig 3.3 B, B', B''). Above results confirm the activity of the overexpressed *Notch-ICD*.

3.2.3 Injection of *aPKC* RNA or *Notch-ICD* does not promote deep cells to express superficial cell markers

Having confirmed the activity of overexpressed *C-aPKC*, *CAAX-aPKC* and *Notch-ICD* in *Xenopus* embryos, the next step was to test their ability to promote superficial cell differentiation. The two types of *aPKC* RNA and the *Notch-ICD* RNA were injected into embryos. Any change in fate was analyzed by *in situ* hybridization at stage 11. *Lac Z* RNA was injected as a lineage label with the

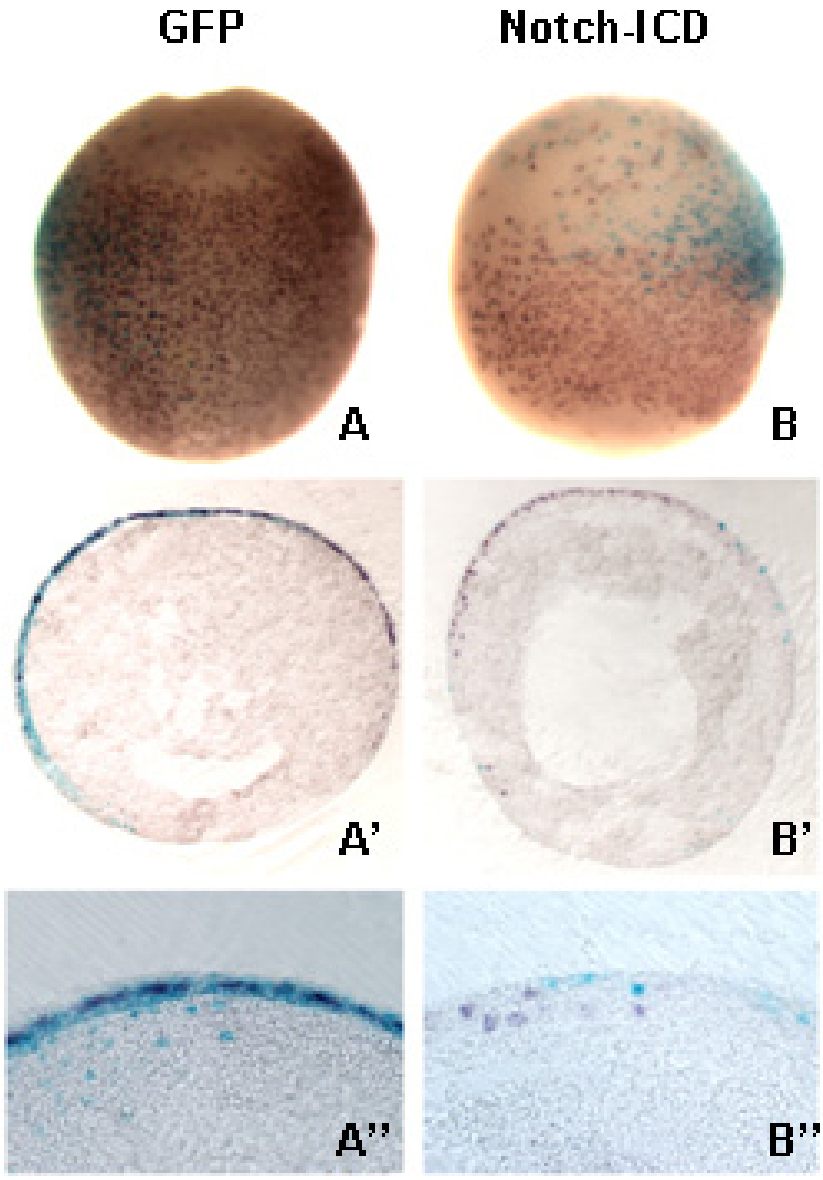


Fig 3.3. *Notch-ICD* expression inhibits expression of *α-Tubulin*. (A-A'-A'') the expression of *α-Tubulin* in *GFP* injected embryos. (B,B',B''). *α-Tubulin* expression in *Notch-ICD* overexpressed embryos. A wholemount and sections are shown from a stage 13 embryo. *LacZ* staining (light blue), *in situ* hybridization (purple). Based on at least 2 independent experiments.

candidate RNA. A batch of 4 superficial markers was used: keratin type I epidermal (*keratin I*), keratin type II epidermal (*keratin II*), ER to nucleus signaling I (*ER I*) and *claudin 4* (Chalmers et al., 2006). In *GFP* control, these genes have specific expression in the superficial layer (Fig 3.4 A-D). In deep cells from *C-aPKC*, *CAAX-aPKC* or *Notch-ICD* overexpressed embryos, which were well stained for Lac Z, there was no superficial markers expression (Fig 3.4 E-H for *C-aPKC*; I-L for *CAAX-aPKC*; M-P for *Notch-ICD*). Superficial markers expression was restricted to the superficial layer, despite positive results from the activity assay, suggesting that C-aPKC, CAAX-aPKC or Notch-ICD are not sufficient to promote superficial cell fate in *Xenopus* deep cells.

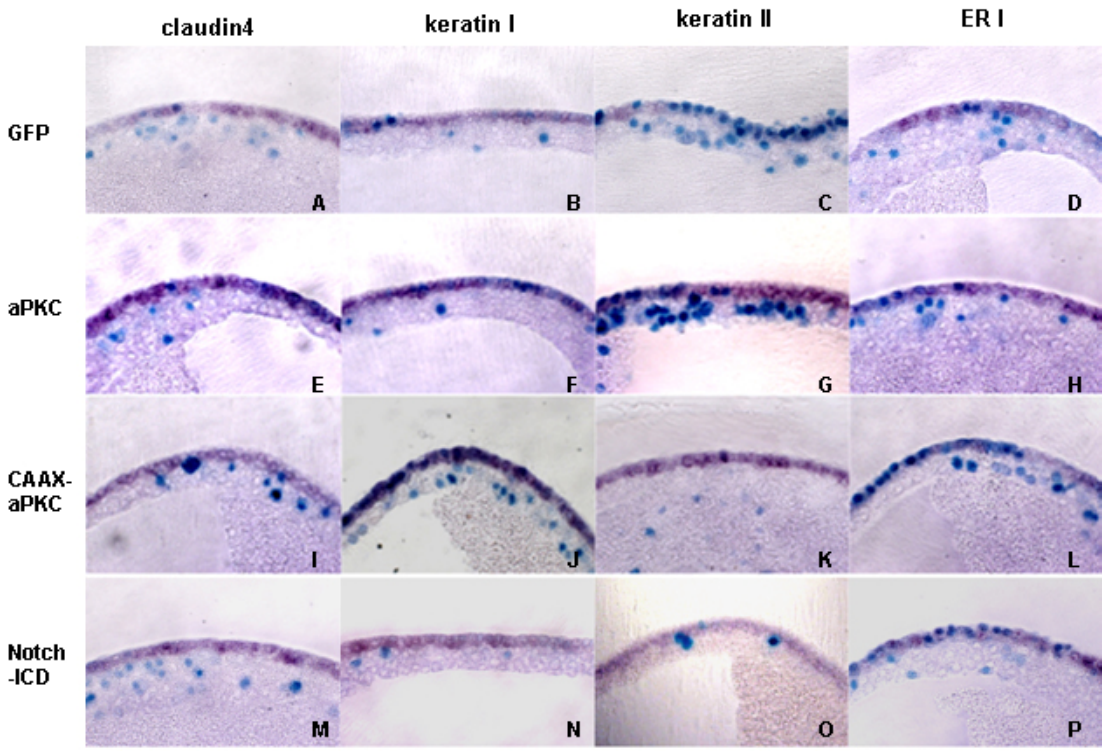


Fig 3.4. C-aPKC, CAAX-aPKC and Notch-ICD did not promote superficial cell marker expression in the deep cells. (A-D) Expression of superficial markers: *claudin4*, *keratin I*, *keratin II* and *ER I* in *GFP* injected embryos. (E-H) Expression of superficial markers: *claudin4*, *keratin I*, *keratin II* and *ER I* in *C-aPKC* injected embryos. (I-L) Expression of superficial markers: *claudin4*, *keratin I*, *keratin II* and *ER I* in *CAAX-aPKC* injected embryos. (M-P) Expression of superficial markers: *claudin4*, *keratin I*, *keratin II* and *ER I* in *Notch-ICD* injected embryos. A section of a stage 12 embryo is shown. LacZ staining (light blue), *in situ* hybridization (purple). Based on at least 3 independent experiments.

3.3 Discussion

3.3.1 aPKC and superficial cell fate

Genetic studies in *Drosophila* reveal that aPKC is responsible for polarity and cell fate specification in neuroblasts (Rolls et al., 2003; Lee et al., 2006). Lgl negatively regulates aPKC, while aPKC directly promotes neuroblast self-renewal (Rolls et al., 2003; Lee et al., 2006). In *Xenopus*, superficial cells are generated at blastula stages by perpendicularly oriented divisions, and aPKC is asymmetrically localized in the apical membrane of cells during the divisions (Chalmers et al., 2003). Although inhibition of Lgl is similar with *Drosophila*, aPKC was not sufficient to promote superficial cell fate (Fig 3.1 to Fig 3.4). This suggests there is a notable difference between asymmetric cell division in *Xenopus* embryonic cells and *Drosophila* neuroblasts. This is consistent with work showing that the orientation of the division may be controlled by different mechanisms in *Xenopus* and *Drosophila* (Strauss et al., 2006).

A recent independent study showed that aPKC overexpression in *Xenopus* embryos is sufficient to promote keratin expression in deep cells (Ossipova et al, 2007). The reason for the difference is not clear, but they use rat PKC and the rat PKC and *Xenopus* PKC may be distinct and result in the difference. Moreover, they investigated the keratin expression at stage 14. This stage is not an easy stage to work with as deep cells normally express low levels of superficial markers. It would be interesting to repeat my experiments at stage 14, to investigate aPKC has an affect on later stage embryos.

To further address questions about asymmetric cell division and superficial cell fate

specification, other members of PAR-aPKC system, such as Par-3 and Par-6 could be investigated. Par-3 and Par-6 could be overexpressed in *Xenopus* embryos at the two-cell stage, and then investigated if they can promote superficial genes expression. This would show if other polarity proteins can promote superficial cell fate

3.3.2 Notch and superficial cell fate

This work shows that in *Xenopus*, Notch overexpression is not sufficient to promote superficial cell fate. This might suggest that Notch does not participate in superficial cell development, but does not rule out a function in the superficial cells. In *Xenopus*, the role of Notch signaling in neurogenesis has been studied in detail (Bellefroid et al. 1998). Research in *Drosophila* and mammals revealed that Notch is involved in asymmetric cell division. In mouse cortical neurogenesis, the mammalian homolog m-Numb remains in the apical daughter cell to suppresses Notch activity. The higher Notch1 activity in the basal cell will contribute to it forming a neural progenitor (Zhong et al., 1996, 1997). In *Drosophila*, Numb also works as a negative regulator of Notch in the cell fate decision of nervous system development (Roegiers and Jan, 2004). In some aspects, the mutual inhibition between Notch and Numb is similar with aPKC/Lgl in neuroblasts' fate decision (Rolls et al., 2003; Lee et al., 2006). However, overexpression of *Notch-ICD* does not change neuroblast asymmetric cell division to produce two neuroblast daughter cells (Seugnet et al., 1997), despite the role that notch plays in this system. This suggests that despite the negative result described here notch could still be involved in *Xenopus* superficial cell development.

There is a different possibility: Notch is not involved in superficial cell specification in *Xenopus*. Since the HES family member ESR6e is specifically expressed in the superficial layers (Chalmers et al., 2002), this hypothesis would suggest that there are other pathways in superficial cells which activate ESR6e. HES member Hairy2 acts downstream of BMP pathway to maintain neural crest progenitors undifferentiating (Nagatomo and Hashimoto, 2007), indicating that the BMP pathway is a possible candidate to activate ESR6e in the superficial layer in *Xenopus*. Testing if BMP is able to promote superficial cell fate will be carried out in Chapter 4.

3.3.3 aPKC, Notch and endocytosis

Numb and Sanpodo are important in external sensory organ development as they regulate Notch activity via endocytosis (Hutterer and Knoblich, 2005; Babaoglan et al., 2009). Conversely, endocytosis is required for Notch activation (Gupta-Rossi et al, 2004). Smith's findings revealed that, in well polarized epithelial cells, the trafficking of Numb is restricted to the basolateral membrane by aPKC phosphorylation (Smith et al, 2007). Therefore, Numb may serve as a bridge between the PAR-aPKC polarity complex and the endocytic machinery. The hypothesis was recently supported by the link between PAR-aPKC system and endocytosis, which is a new role for the complex (Nishimura and Kaibuchi, 2007).

Neither PAR-aPKC nor Notch-ICD promotes superficial cell fate specification by promoting the superficial genes expression. However, they may help localize cell fate

determinant molecules by regulating endocytosis. To confirm this hypothesis, gain-and-loss of function experiments on membrane trafficking proteins could be carried out. For example, blocking endocytic proteins such as the ESCRT proteins (Dukes et al., 2008), and investigating the regulation of superficial or deep cell fate.

This data did not indicate that aPKC or Notch have the activity to promote differentiation of the first epithelial cell type in *Xenopus* so it was decided to stop working with these two proteins and focus on other candidates.

4 Chapter IV BMP4 and *Xenopus* superficial cells specification

4.1 Introduction

BMPs belong to TGF-β superfamily of secreted signaling proteins, were first identified as the molecules with the ability to induce ectopic bone and cartilage formation in rodents (reviewed in Hogan, 1996). As discussed in the general introduction, the BMPs transducte their signals through the heterotetrameric dimers consisting of type I (ALK2, 3, 6) and type II (BMPRII) serine/threonine kinase transmembrane receptors. Subsequently BMPs activate Smads 1, 5 and 8 to mediate intracellular signaling in collaboration with the common Smad4 (Attisano L, et al, 2002).

Further research revealed BMPs play critical roles in embryogenesis and tissue homeostasis (De Robertis and Kuroda, 2004; Varga and Wrana, 2005). In the *Xenopus* gastrula, the cells localized at different sides of the ectoderm will follow different cells fates: ventral side cells will differentiate into epidermis, while the cells from the dorsal side will become nervous system (Hemmati-Brivanlou and Melton, 1997). BMP4 is a key promoter of epidermal differentiation (Wilson and Hemmati-Brivanlou, 1995). Moreover, BMP activates a number of transcription factors including Grhl1 which is specifically expressed in the superficial layer of the epidermis (Tao et al., 2005). However, as mentioned in the main introduction, the epidermis has two distinct layers: the outer superficial layer, and inner deep layers (Chalmers et al., 2002). However, this fact was largely ignored by previous research, so that two layers were often analyzed as a unit, but it is very possible they are promoted by different

factors and if BMP4 promotes both layers of the epidermis remains unknown. This chapter will investigate the role of BMP4 in promoting superficial and deep cell fate.

4.2 Results

4.2.1 BMP4 promotes superficial gene expression but inhibits deep gene expression

The strategy from Chapter III was used to investigate BMP4's role in *Xenopus* superficial cell differentiation. First, the overexpressed molecules activity was confirmed. Then any effect on superficial or deep cell genes was analyzed

4.2.1.1 The injected *Bmp4* and *Noggin* RNA is active in *Xenopus*

To check if overexpressed *Bmp4* and *Noggin* RNA, which are both used in later sections, were active in *Xenopus* embryos, inhibition/expansion of neural development was used as an assay (Wilson et al., 1995). Three groups of RNA were injected into *Xenopus* embryos at the 2-cell-stage, *GFP* (1ng/embryo), *Bmp4* (1ng/embryo) and *Noggin* (0.1ng/embryo). *Lac Z* RNA (0.5 ng/embryo) was injected as a lineage label. The activity assay was carried out by *in situ* hybridization with two different markers: epidermal marker *Keratin II* and neural plate marker *Sox3*. The embryos at stage 16 showed that there were significant differences between three groups. In the *GFP* control, the embryos had *Keratin II* expression in the epidermis but not in the neural plate (Fig 4.1 A). In contrast, *Sox3* was only expressed in neural

plate (Fig 4.1 D). In *Bmp4* overexpressed embryos stained for Lac Z, the developmental patterning was dramatically changed. *Keratin II* staining covered almost the whole embryos (Fig 4.1 B). The expression of *Sox3* was significantly reduced, showing an inhibition of neural plate formation (Fig 4.1 E). *Noggin* overexpressed embryos' developmental patterning was changed as well, but as expected it was opposite to *Bmp4*. There was reduced staining of *Keratin II* (Fig 4.1 C) and *Sox3* expression was significantly expanded (Fig 4.1 F): most surfaces of embryos were covered by *Sox3* staining. Based on these results, it was concluded that the overexpressed *Bmp4* and *Noggin* were both active in *Xenopus* embryos.

4.2.1.2 *Bmp4* promotes deep cells to express superficial markers

After confirming *Bmp4* activity in *Xenopus* embryos, the next step was to examine the ability of BMP4 to promote superficial cell differentiation. *Bmp4* RNA was injected into embryos for *in situ* hybridization at stage 11. *Lac Z* RNA was injected as a lineage label. A batch of three superficial markers was used: keratin type I epidermal (*keratin I*), keratin type II epidermal (*keratin II*), and *claudin 4* (Chalmers et al., 2006). In the *GFP* control, these genes had specific expression in the superficial layer of the epidermal ectoderm (Fig 4.2 D, E, F). In *Bmp4* overexpressing embryos, which were well stained for Lac Z, superficial marker expression was not limited to the superficial layer. The deep cells in "the injected area" expressed superficial markers as well (Fig 4.2 A, B, C). These results showed that BMP4 was sufficient to promote superficial cell fate in deep cells of *Xenopus*.

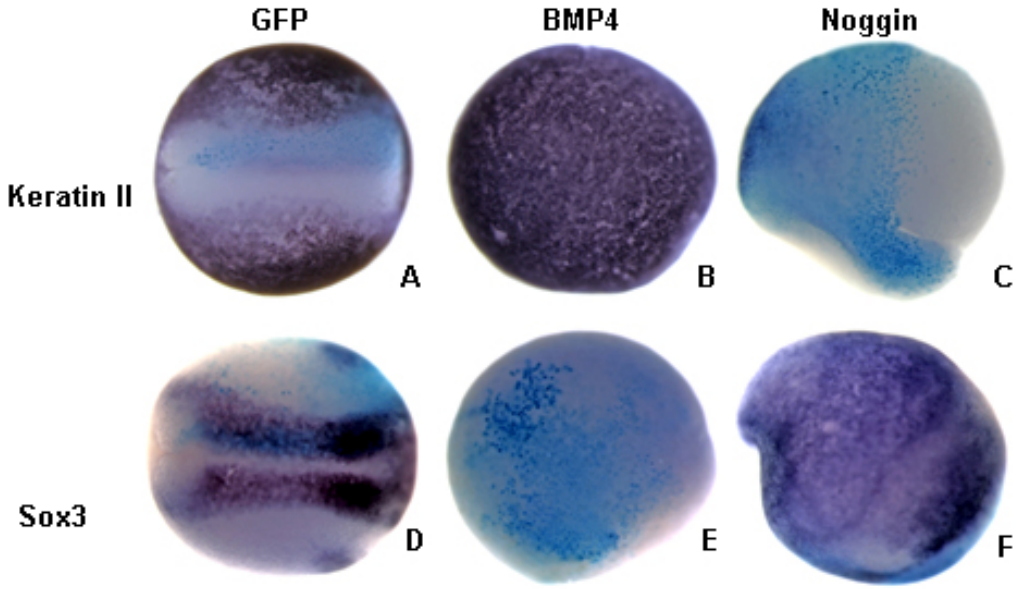


Fig 4.1 BMP4 expands and Noggin inhibits the epidermis. (A) Expression of *Keratin II* in embryos injected with *GFP*. (B) Expression of *Keratin II* in embryos injected with *Bmp4*. (C) Expression of *Keratin II* in embryos injected with *Noggin*. (D) Expression of *Sox3* in embryos injected with *GFP*. (E) Expression of *Sox3* in embryos injected with *Bmp4*. (F) Expression of *Sox3* in embryos injected with *Noggin*. A wholemount stage 16 embryo is shown. LacZ staining (light blue), *in situ* hybridization (purple). Based on at least 3 independent experiments.

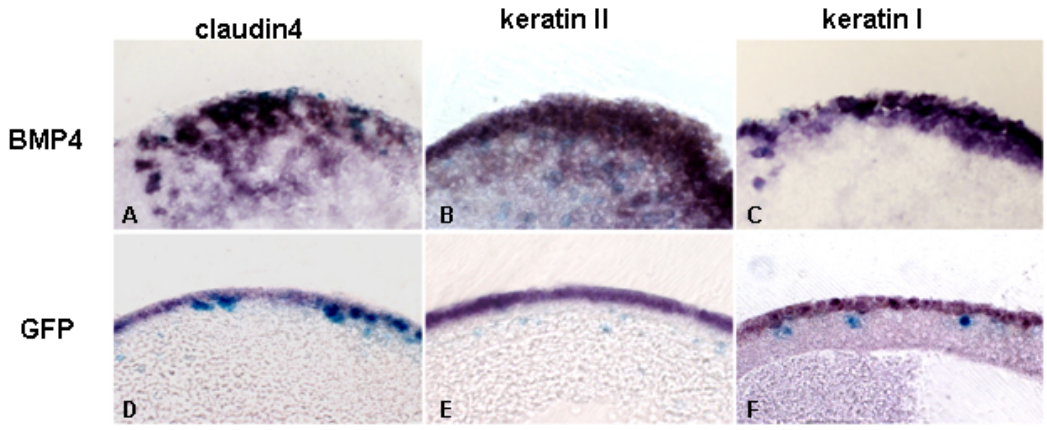


Fig 4.2 BMP4 promotes deep cells to express superficial cell markers. (A-C) The expression of superficial markers: *claudin4*, *keratin I*, *keratin II* in embryos injected with *Bmp4*. (D-F) The expression of superficial markers: *claudin4*, *keratin I*, *keratin II* in embryos injected with *GFP*. A section of a stage 11 embryo is shown. LacZ staining (light blue), *in situ* hybridization (purple). Based on at least 3 independent experiments.

4.2.1.3 *Alk2* overexpression promotes deep cells to express superficial cell markers

The previous section suggests that activating the BMP pathway is sufficient to promote superficial cell fate in *Xenopus* embryos. This predicts that activating BMP4 receptors should induce the same effect. To test the prediction, constitutively active BMP4 receptor ALK2 (Suzuki et al., 1997) was used to carry out the analysis. *Alk2* RNA (0.5ng/embryos) was injected into embryos, and differentiation was analyzed in the same way as with *Bmp4*. Keratin type I epidermal and *claudin 4* (Chalmers et al., 2006) were used to probe superficial differentiation. In *GFP* control, these genes had specific expression in the superficial layer of the epidermal ectoderm (Fig 4.3 C, D). In *Alk2* overexpressed embryos, which were well stained for Lac Z, expression of superficial markers was found in both the superficial layer and the deep cells in the injected area (Fig 4.3 A, B), consistent with the *Bmp4* results. Thus, it was confirmed that activating the BMP pathway can promote superficial cell fate.

4.2.1.4 Injection of BMP4 RNA inhibits deep genes expression

BMP4 signalling has a well established role in promoting epidermal cell fate in *Xenopus* embryos (Wilson, 1995). However, the former work has mostly not considered that the *Xenopus* epidermis consists of two layers. Superficial cells differentiate early while the deep cells remain undifferentiated until later stages (reviewed by Chalmers et al., 2003). Furthermore, a number of genes are specifically expressed in the different layers (Chalmers et al., 2006). The former section confirmed that BMP4 is sufficient to promote superficial layer differentiation in *Xenopus*. It is also necessary to investigate BMP4's effect on

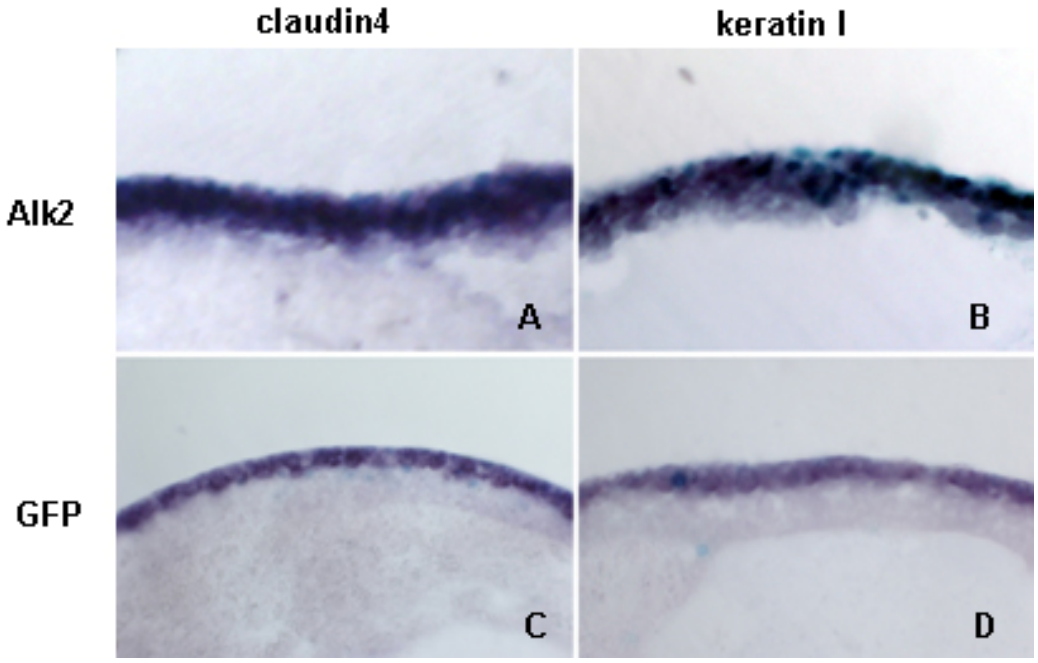


Fig 4.3 ALK2 promotes deep cells to express superficial markers. (A-B) The expression of superficial markers: *Claudin4* and *keratin I* in embryos injected with *Alk2*. (C-D) The expression of superficial markers: *Claudin4* and *keratin I* in embryos injected with *GFP*. A section of a stage 11 embryo is shown. LacZ staining (light blue), *in situ* hybridization (purple). Based on at least 2 independent experiments.

deep cell specific genes expression.

Similar to the superficial cell fate assay, *in situ* hybridization was used to examine the expression of deep cell genes. *Bmp4* RNA was injected to investigate deep gene expression by *in situ* hybridization at stage 11. *Lac Z* RNA was injected as a lineage label with *Bmp4* RNA. Hyaluronan synthase I and prothymosin alpha (Chalmers et al., 2006) were used as deep cell markers. In *GFP* control embryos (Fig 4.4 A, B) and the control side of *Bmp4* injected embryos (Fig 4.4 C, D), these genes were specifically expressed in the deep layer of the epidermal ectoderm. In the *Bmp4* overexpressed side, which was well stained for Lac Z, expression of deep gene markers were dramatically inhibited, and there was no staining in either the superficial layer or deep layer (Fig 4.4 E, F). This shows that in addition to inducing superficial cell fate, BMP4 was able to inhibit deep gene expression.

4.2.1.5 *Alk2* overexpression does not inhibit deep genes expression

By overexpressing *Bmp4* and *Alk2*, it was confirmed that the BMP4 pathway plays a role in promoting superficial cell differentiation. Moreover, overexpression of *Bmp4* did not promote, but actually inhibite deep genes expression. ALK2's effect on deep gene expression was then investigated.

Alk2 RNA (0.5 ng/ embryo) was injected to analyse deep genes expression by *in situ* hybridization at stage 11. *Lac Z* RNA was injected as a lineage label with *Alk2* RNA. Hyaloronan synthase I and prothymosin alpha were used to probe deep genes.

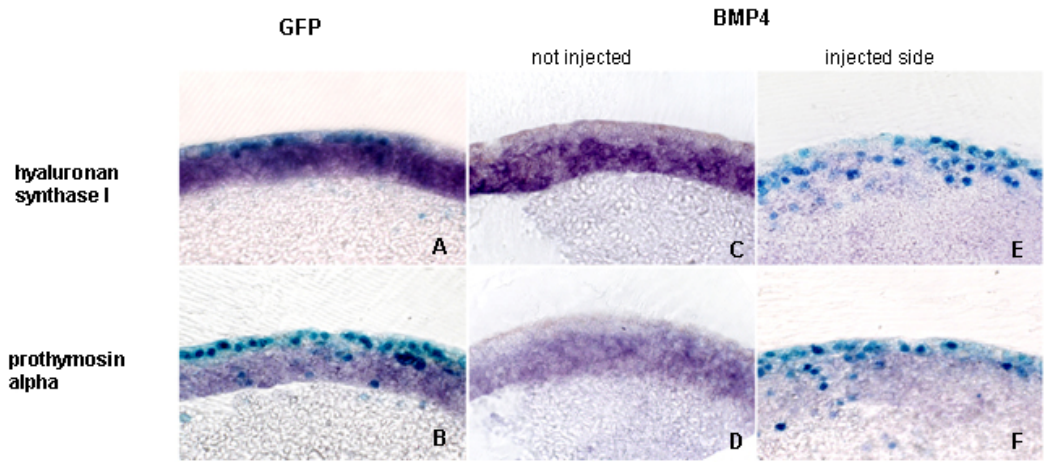


Fig 4.4 BMP4 inhibits deep gene expression. (A-B) The expression of deep markers in embryos injected with *GFP*. (C-D) The expression of deep markers in the non injected side of embryos injected with *Bmp4* at the other side. (E-F) The expression of deep markers in the injected side of embryos injected with *Bmp4*. A section of a stage 11 embryo is shown. LacZ staining (light blue), *In situ* hybridization (purple). Based on at least 3 independent experiments.

In GFP control these genes were specifically expressed in the deep layer of the epidermal ectoderm (Fig 4.5 B, D). Surprisingly, in *Alk2* overexpressed embryos, the deep genes expression was the same as GFP but not as *Bmp4*, expression of deep markers was present in the deep layers (Fig 4.5 A, C). Thus, deep gene marker expression was not changed by ALK2.

The data presented above shows that BMP4 is sufficient to promote superficial cell differentiation, but not promote deep cell fate and may even inhibit it.

4.2.2 BMP4 signaling is required for superficial but not deep cell fate

The above observations suggest that BMP4 signalling is involved in superficial cell differentiation. To address if BMP4 signalling is required for superficial cell differentiation, Noggin was introduced into the system. Noggin is well known as a BMP antagonist which acts by directly binding to BMPs (Zimmerman et al., 1996). Overexpression of *Noggin* can inhibit BMPs pathways including BMP4 signalling.

4.2.2.1 Injection of *Noggin* inhibits superficial gene expression

The effect of Noggin on superficial cell differentiation was investigated by the same method as BMP4. *Noggin* RNA (0.1 ng/embryo) was injected to analyse superficial cell differentiation by *in situ* hybridization at stage 11. *Keratin II* and *claudin 4* used to probe superficial genes (Chalmers et al., 2006). In *GFP* injected embryos, these genes specifically expressed in the superficial layer of the epidermal ectoderm (Fig

4.6 A, B). In *Noggin* embryos, which were well stained

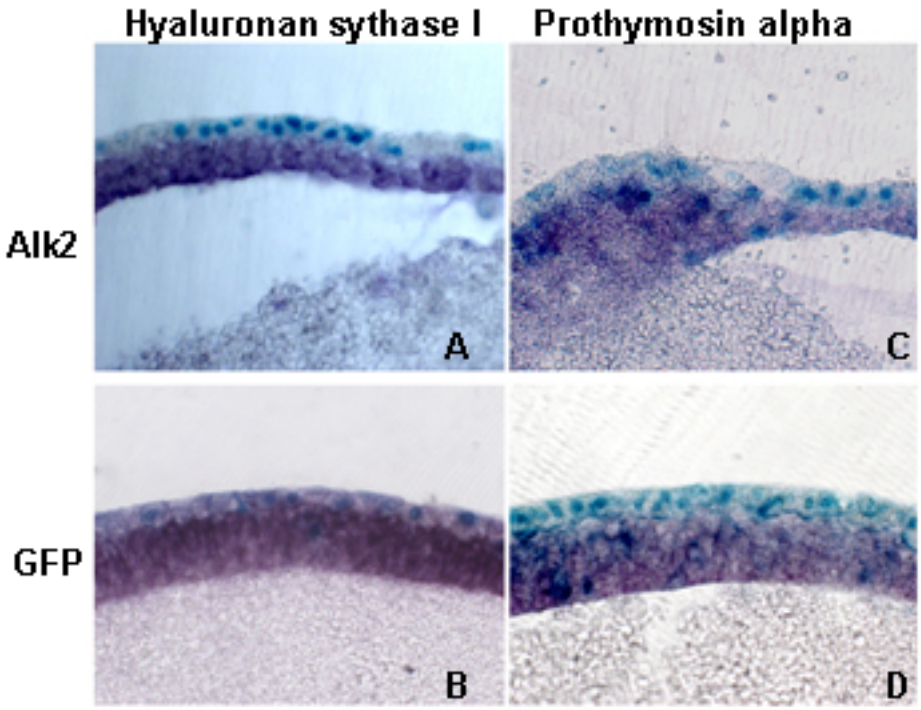


Fig 4.5 ALK2 does not inhibit deep gene expression. (A) The expression of the deep marker hyaloronan synthase I in embryos injected with *Alk2*. (B) The expression of the deep marker hyaloronan synthase I in embryos injected with *GFP*. (C) The expression of the deep marker Prothymosin alpha in embryos injected with *Alk2*. (D) The expression of the deep marker Prothymosin alpha in embryos injected with *GFP*. A section of a stage 11 embryo is shown. LacZ staining (light blue), *in situ* hybridization (purple). Based on at least 3 independent experiments.

for Lac Z, superficial genes expression was dramatically inhibited and there was no superficial marker expression found (Fig 4.6 C, D). It was concluded that BMP signaling is required for superficial cell fate in *Xenopus*.

4.2.2.2 Injection of *Noggin* RNA does not inhibit deep cell makers

To investigate Noggin's effect on deep genes, *Noggin* RNA (0.1 ng/embryo) was injected to analysis deep gene expression by *in situ* hybridization at stage 11. *Lac Z* RNA was injected as a lineage label with *Noggin* RNA. Two deep genes were investigated: hyaloronan synthase I and prothymosin alpha. In *GFP* control embryos, these genes specifically expressed in the deep layer of the epidermal ectoderm (Fig 4.4 and Fig 4.7 A, B). Unlike *Bmp4* embryos (Fig 4.4), deep genes' expression was not changed by *Noggin* overexpression was the same as the *GFP* control (Fig 4.7 C, D). The result demonstrates that, unlike superficial genes, deep genes do not require BMPs for their activation. In addition, Noggin did not cause superficial cells to express deep cell markers suggesting that inhibiting BMP is not enough to activate deep cell genes.

4.2.3 BMP4 promotes expression of a range of superficial cell junction proteins, but does not induce junction assemble in deep cells

Keratin I, *Keratin II*, and tight junction gene *claudin 4* were used in this project as superficial markers. Although BMP4 and ALK2 can promote those superficial markers expression in deep cells, they do not represent all the cell junction proteins which are typically expressed in *Xenopus* superficial cells. Two other tight junction

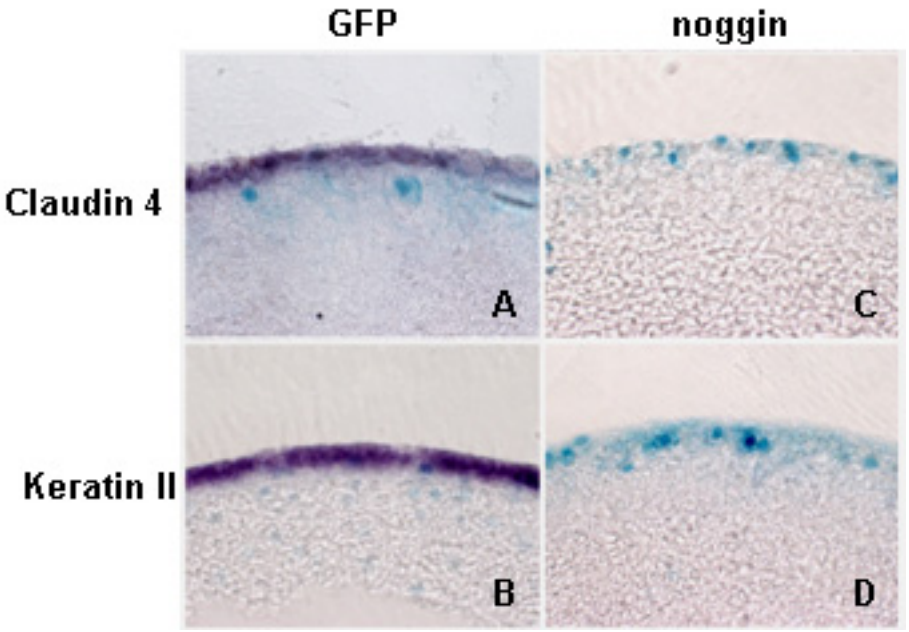


Fig 4.6 Noggin inhibits superficial gene expression. (A-B) The expression of superficial markers: *claudin4* and *keratin II* in embryos injected with *GFP*. (C-D) The expression of superficial markers: *claudin4* and *keratin II* in embryos injected with *Noggin*. A section of a stage 11 embryo is shown. LacZ staining (light blue), *in situ* hybridization (purple). Based on at least 3 independent experiments.

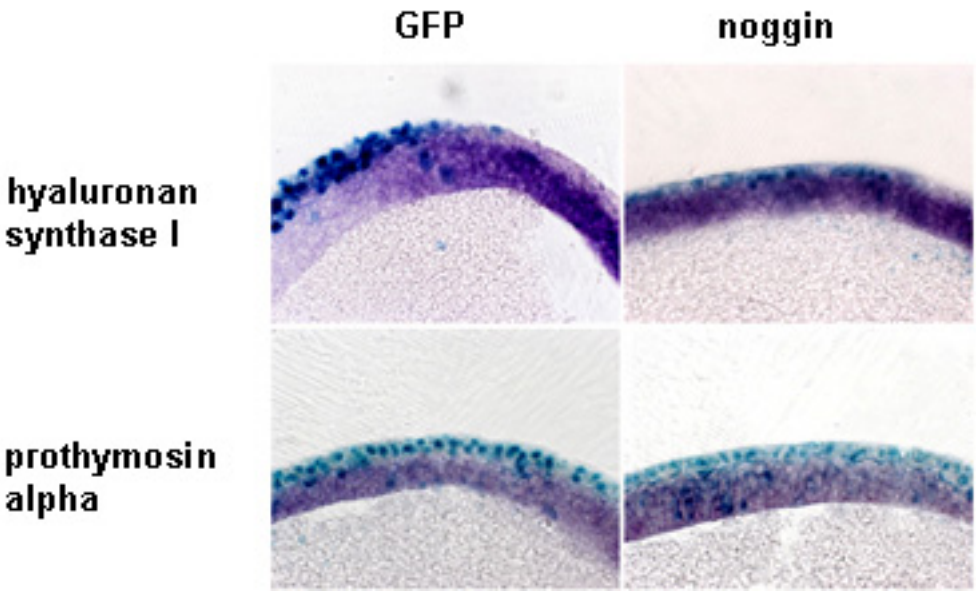


Fig 4.7 Noggin does not inhibit deep gene expression. (A-B) The expression of deep markers: hyaloronan synthase I and Prothymosin alpha in embryos injected with *GFP*. (C-D) The expression of deep markers: hyaloronan synthase I and Prothymosin alpha in embryos injected with *Noggin*. A section of a stage 11 embryo is shown. LacZ staining (light blue), *in situ* hybridization (purple). Based on at least 3 independent experiments.

proteins, Occludin and Cingulin, were selected to confirm the role of BMP4 signalling function in superficial cell differentiation.

Bmp4 and *gfp* RNA were injected respectively to analyse *Cingulin* and *Occludin* expression. *In situ hybridization* was presented at Stage 16 as the genes could not be detected at earlier stages. In *GFP* control, *Occludin* has specific expression in the superficial layer of the epidermal ectoderm (Fig 4.8 A). In *Bmp4* overexpressed embryos, *Occludin* expression was promoted in both superficial cell and the deep cells (Fig 4.8 C). *Cingulin* was expressed in both superficial and deep layers in *GFP* controls (Fig 4.8 B) and it was enhanced in both layers by *Bmp4* overexpressed (Fig 4.8 D). This suggests that BMP4 signaling is sufficient to promote expression of a range of epithelial junction proteins.

Cell polarity is a key feature of superficial cells in *Xenopus*. BMP4 promotes superficial cell fate and the expression of junction proteins. However, it is not clear if BMP4 can promote polarization and junction formation in the deep cells. The cell polarity protein aPKC, stained with the c-20 antibody, was used to assess apical-basolateral cell polarity establishment in the deep cells. *Bmp4* and *GFP* RNA were injected into embryos which were sectioned at stage 11. Cell polarity was analyzed by IF. In *GFP* embryos, aPKC (c-20) was localized in the apical domain of *Xenopus* superficial cells (Fig 4.9 A, B). In *Bmp4* embryos, aPKC (c-20) the apical domain localization was changed in the superficial cells. It was difficult to find a clear aPKC (c-20) stained apical domain. In addition, brighter aPKC (c-20) staining was noticed in cytoplasm (Fig 4.9 C, D). This suggests that BMP4 may increase aPKC expression, but inhibits aPKC localization to the apical membrane domain. This means that aPKC can not be used to show if BMP4 induces cell polarization.

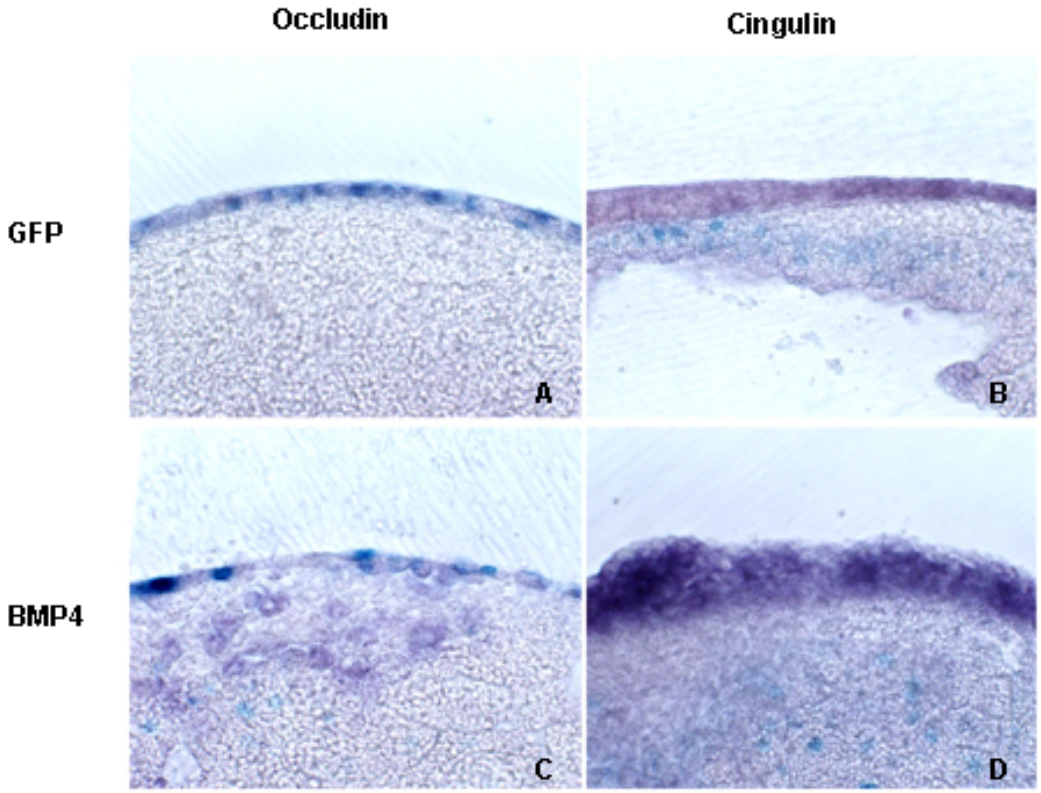


Fig 4.8 BMP4 promotes expression of a range of junction proteins in the deep cells. (A-B) The expression of superficial markers: *Occludin* and *Cingulin* in embryos injected with *GFP*. (C-D) The expression of superficial markers: *Occludin* and *Cingulin* in embryos injected with *Bmp4*. A section of a stage 16 embryo is shown. LacZ staining (light blue), *in situ* hybridization (purple). Based on at least 3 independent experiments.

A tight junction protein ZO1 was used to analyze the capacity of BMP4 to promote junction formation in the deep cells. In *GFP* embryos, ZO1 was localized in the junction region of the superficial cells (Fig 4.9 E, F). If BMP4 could cause junction formation, there would be ZO1 positive junctions in deep cells. Actually, in *Bmp4* embryos, ZO1 localization was not changed. Similar to aPKC (c-20), there was more expression of ZO1 in cytoplasm (Fig 4.9 G, H). In summary, BMP4 was not sufficient to promote assembly of tight junction proteins in *Xenopus* superficial cells.

4.2.4 BMP signaling is active in both layers

The above work demonstrated the role of BMP4 signalling in promoting superficial fate in *Xenopus*. However, where the difference between superficial layer and deep cells occurs in the BMP4 pathway remains unknown. Active Smad1/5/8 complex is crucial for BMP4 signalling. It delivers signaling from the BMP4 receptors to the nucleus, and then initiates downstream cascades by turning on different transcriptional factors (Attisano L, et al, 2002). The amount of active BMP4 signalling was assayed by phospho- Smad1/5/8 antibody. *Bmp4* RNA was injected into embryos which were sectioned at stage 11. *GFP*-injected embryos were used as the control. Comparing the localization of Smad1/5/8 in the sections, there was phospho-Smad1/5/8 in both layers of the two groups. The brightness of the staining in BMP4 sections (Fig 4.10 C, D) was much stronger than the *GFP* embryos (Fig 4.10 A, B), but there was no Smad1/5/8 enrichment in the superficial layers (Fig 4.10 A-D). The results indicated that there was no difference between active Smad1/5/8 levels in the superficial and deep layers.

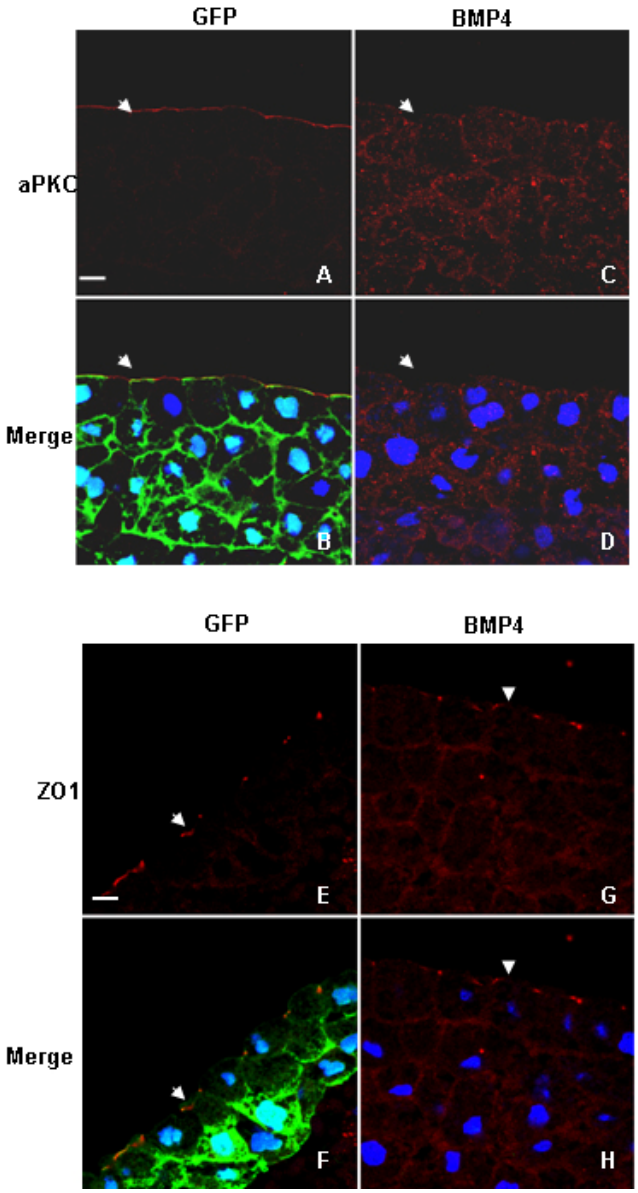


Fig 4.9 BMP4 changes aPKC but not ZO-1 localisation. (A-B) The localisation of aPKC (c-20) in embryos injected with *GFP*. (C-D) The localisation of aPKC (c-20) in embryos injected with *Bmp4*. (E-F) The localisation of ZO-1 in embryos injected with *GFP*. (G-H) The localisation of ZO-1 in embryos injected with BMP4. aPKC (C-20) (red), ZO1 (red), GFP (Green), DNA (Blue). Sectioning and staining was carried out at Stage 11. Scale bar equals 10 μm. Based on at least 3 independent experiments.

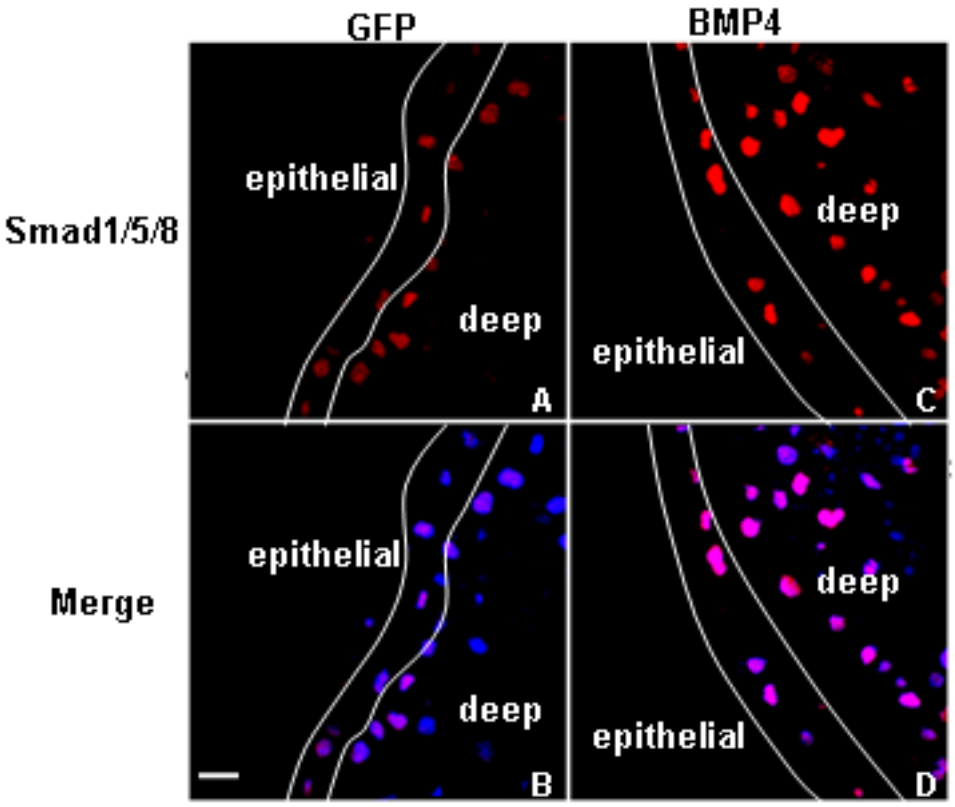


Fig 4.10 Overexpressed *Bmp4* activates more Smad1/5/8 but there was no difference between superficial and deep layers. (A-B) The expression of Smad1/5/8 in embryos injected with *GFP*. (C-D) The expression of Smad1/5/8 in embryos injected with *Bmp4*. Section and staining was carried out at Stage 11. Smad1/5/8 (red), DNA (Blue). Scale bar equals 10 μm. Based on at least 3 independent experiments.

4.2.5 BMP4 target genes are expressed by the deep cells

The Phospho-Smad1/5/8 staining illustrated there was no difference in Smad1/5/8 signal between the two layers. It raises the possibility that transcription factors downstream of Smad1/5/8 may promote the fate difference between superficial layer and deep cells. The expression of known BMP target genes was analysed. The superficial layer and deep layers were isolated from normal *Xenopus* embryos at stage 11-12. RNA was extracted to perform RT-PCR. Ornithine decarboxylase (ODC) was used as the ubiquitous control. Three established BMP4 targets, *Msx1* (Suzuki et al., 1997), *Ap2* (Luo et al., 2002) and *Vent2* (Onichtchouk et al., 1996) were found to be expressed in both superficial and deep layers (Fig 4.11). This demonstrates that some BMP target genes are expressed by the deep cells.

Grhl1 and *Grhl3* have stronger expression in the superficial cells than deep cells (Chalmers et al., 2006; Tao et al., 2005 Fig 4.11) and Grhl3 can promote superficial cell fate in the deep cells of the epidermis (Chalmers et al., 2006). Grhl1 is activated by BMP signalling (Tao et al., 2005), but it is not known if Grhl3 it is also activated by BMP signaling.

A dissociated animal cap assay was used to establish if *Grhl3* is induced by BMP4 signaling. The protocol is described in Fig 4.12 A. *Grhl3* expression was found to be up-regulated by BMP4 signaling (Fig 4.12 B). *Grhl1* and *Msx1* were also up-regulated, as expected (Suzuki et al., 1995; Tao el al., 2005). In conclusion, there are two types of BMP target genes, the one activated in both layers and the other mainly activated in the superficial layer.

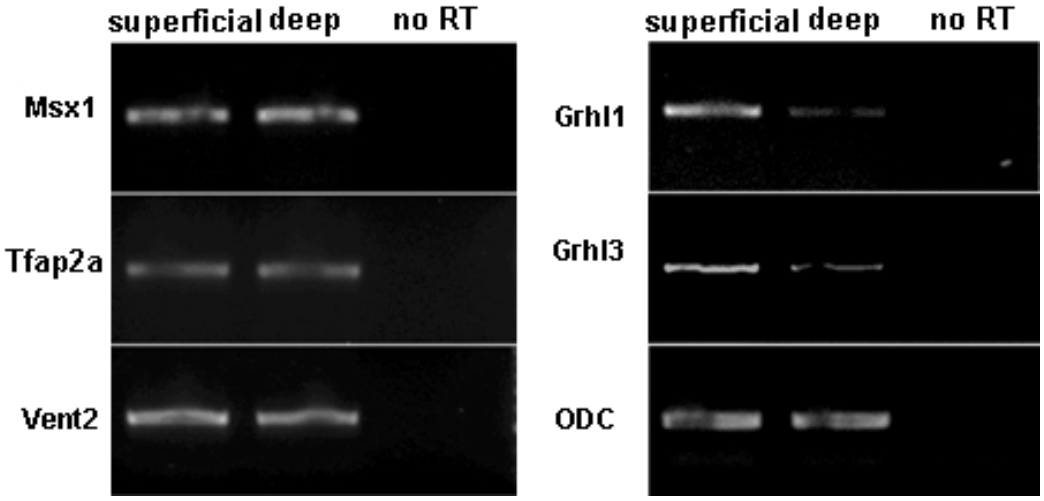


Fig 4.11 Expression profile of the superficial and deep cells. RT-PCR analysis of *Msx1*, *Ap-2*, *Vent2*, *Grhl1* and *Grhl3* expression in superficial and deep cells. RNA was extracted from superficial cells and deep cells from stage 11-12 embryos. Based on at least 3 independent experiments.

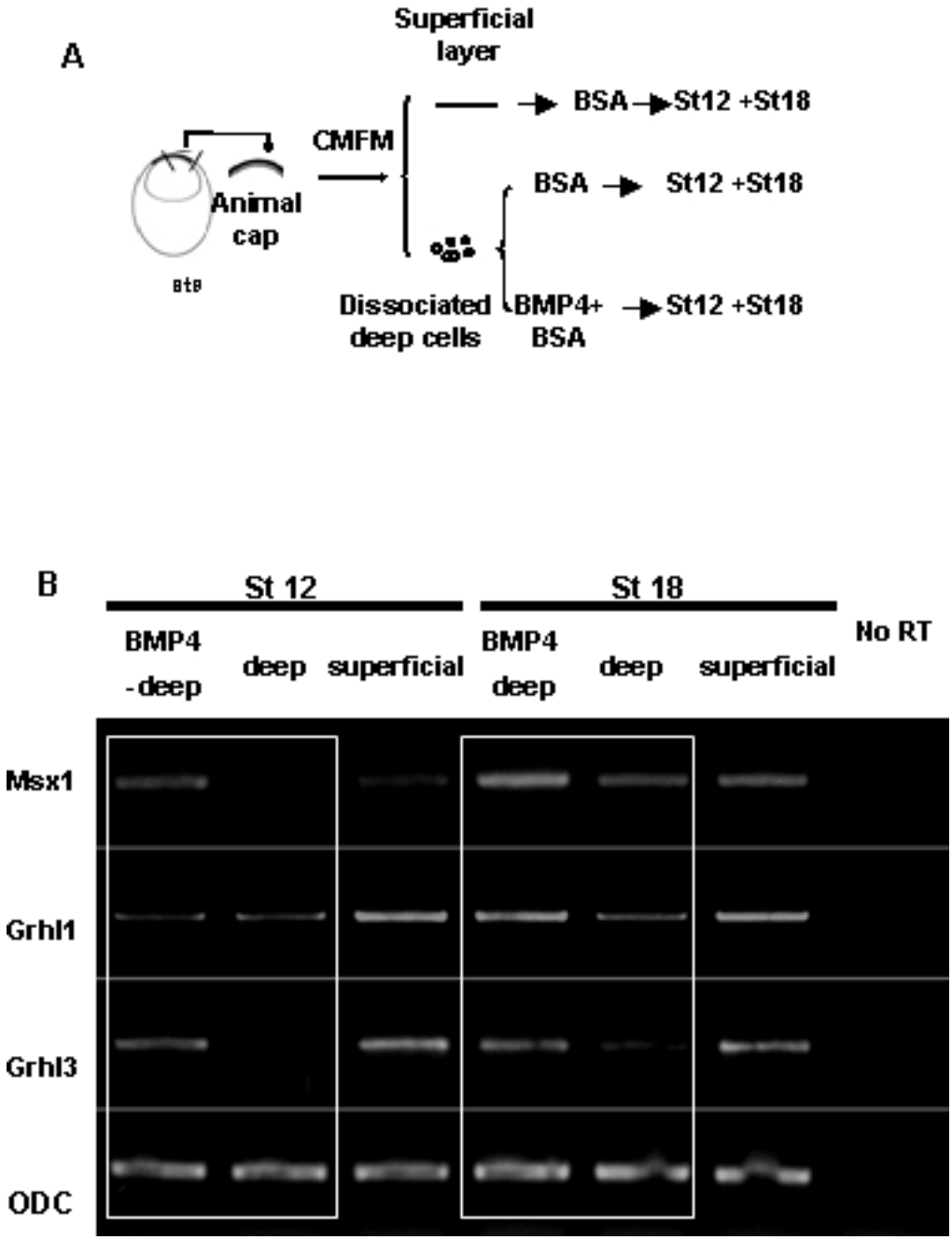


Fig 4.12 BMP4 up-regulates *Msx1*, *Grhl1* and *Grhl3* in the deep cells. (A) Cell dissociation and BMP4 induction assay. (B) *Grhl3*, *Grhl1* and *Msx1* expression in controls or after BMP4 treatment. Based on at least 3 independent experiments.

4.3 Discussion

4.3.1 A new model for the role of BMP signalling in the early epidermis

It is well established that activating the BMP4 cascade leads to epidermal specification by activating different transcriptional factors, but most previous research has ignored one thing: that not all epidermis cells follows the same cell fate, some of them form a superficial protective layer while the inner cells give rise to adult skin (reviewed by Chalmers et al., 2002; 2006). We have analysed the role of BMP in the superficial and deep cells and based on this data we propose a model for epidermal differentiation (Fig. 4.13). An overview of the model will be given in this section, and then individual aspects will be discussed in more detail in following sections.

BMP signaling, via nuclear Smad 1/5/8, activates a subset of BMP target genes in both superficial and deep layers, promoting induction of the epidermis. However, the full range of target genes is only activated in the superficial layer. The expression of superficially restricted genes, such as *Grhl1* and *Grhl3*, then drive expression of differentiation markers such as *Keratins* and *Claudin4* in the superficial cells. Blocking BMP4 does not effect deep genes expression, suggesting the pathway controlling deep gene expression is independent of BMP4. If BMP signalling is inhibited in the epidermis, the deep genes would still be expressed, however, key epidermal genes, such as *Msx1*, *Ap-2* and *Vent-2*, would be lost from the superficial and deep layers. This would mean that epidermal induction would not occur, consistent with the observation that inhibiting BMP signalling in animal caps blocks epidermal induction and allows neural induction to occur. An inhibitor(s) of

differentiation maybe expressed by the deep cells to block the pathway downstream of *Msx*/*Ap-2*/*Vent-2* and stop activation of *Grhl1* and *Grh3*, and so restrict deep cells differentiate to the superficial cells. This inhibitor was designated as Gene X. However, there remain lots of details to be clarified which will be discussed below.

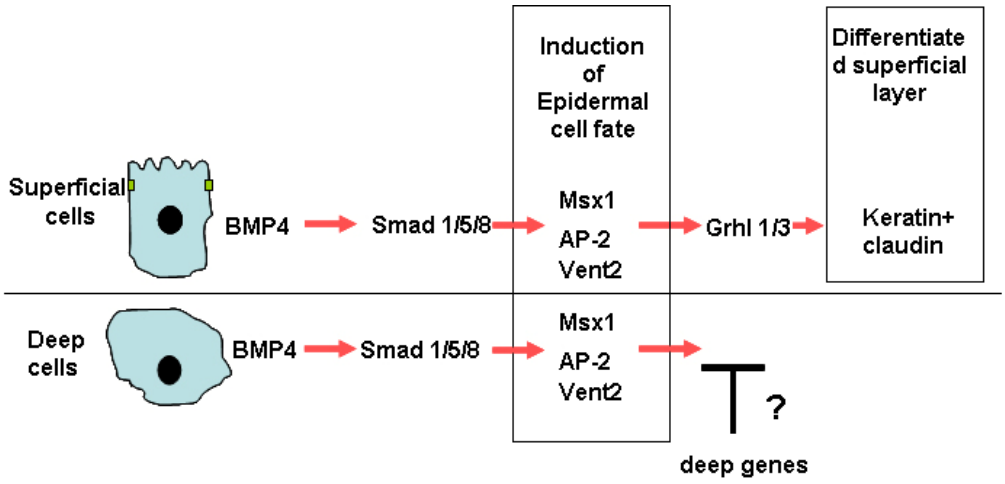


Fig 4.13 A schematic diagram explaining the role of BMP signalling in the superficial and deep cells of the *Xenopus* epidermis. BMP signalling activates a subset of BMP target genes in both layers, promoting induction of the epidermis. However, the full range of target genes is only activated in the superficial layer. The expression of superficially restricted genes, such as *Grhl1* and *Grhl3*, then drive expression of differentiation markers such as *keratins* and *claudin4* in the superficial cells. If BMP signalling is inhibited, the deep genes would still be expressed, however key epidermal genes, such as *Msx1*, *Ap-2* and *Vent*, would be lost from the superficial and deep layers. This would mean that epidermal induction would not occur, consistent with the observation that inhibiting BMP signalling in animal caps blocks epidermal induction and allows neural induction to occur. It is tempting to speculate that an inhibitor(s) of differentiation maybe expressed by the deep cells which would stop activation of *Grhl1* and *Grh3* and so restrict differentiation to the superficial cells.

4.3.2 BMP molecules and their receptors

4.3.2.1 ALK3

ALK2 is sufficient to promote superficial cell fate, but does not effect deep gene expression. In contrast BMP4 does inhibit deep cell genes. The difference may due to ALK2 activating both BMP4 and Activin pathways (Ebner et al., 1993; Macias-Silva et al., 1998). ALK2 regulation of activin signalling may counteract that from BMP4. The results also suggests that more than one receptor is involved in the BMP response. In *Xenopus*, ALK4 receptor can not affect BMP4-mediated epidermal induction, although it can block mesoderm induction by competing for ActRIIB (Chang et al., 1997). Thus, the type I receptor ALK3 may play a role in inhibiting deep genes. Similar with Alk2, ALK3 may be recruited by BMP4 and then activate Smad1/5/8 to promote the superficial gene expression and more interestingly ALK3 may inhibit deep genes. The hypothesis could be tested by overexpressing ALK3 into the embryos, then screening with both superficial and deep markers.

4.3.2.2 Alternative BMP4 signalling pathways

Two signaling pathways are activated by signaling through the BMP4 receptor complex. The first one involves phosphorylation of the BMP4 effector Smad1. The second pathway goes via TGF-β-activated kinase (TAK1), which is a mitogen-activated protein kinase kinase kinase (MAPKKK) (Yamaguchi *et al.,* 1995). Via TAK1 kinase, BMP4 inhibits MAPK activity in *Xenopus* gastrula ectoderm. This inhibition is not dependent on protein synthesis, but via inhibitory crosstalk between TAK1 and MAPK pathways (Goswami et al., 2001). In *Xenopus*, TAK1 is essential in

establishing ventral mesoderm in response to BMP4 (Shibuya et al., 1998). On the other hand, MAPK is necessary for the neural fate in response to endogenous signals (Uzgare *et al.,* 1998). There is a threshold for MAPK to inhibit Smad1 activity or activate other molecules that could promote neural specification (Goswami et al., 2001). In the *Xenopus* ventral ectoderm, BMP4 prevents MAPK activity from increasing to such a threshold (Goswami et al., 2001). In mammalian cells, the interaction between MAPK and Smad1 was also observed (Kretzschmar et al., 1997). Apart from MAPK, the BMP4/TAK1 pathway also activates JNKs (Shirakabe et al., 1997).

Whether the effectors responding to TAK1 are involved in superficial cell differentiation remains unknown. However, the TAK1 pathway may serve as a link between BMP and MAPK. Knocking down TAK1 by MOs may show if TAK1 is required for activating superficial cell genes or inhibiting deep cell genes.

4.3.3 The pathway downstream of BMP4

4.3.3.1 Msx1 and Msx2

Msx1 is well established as one of BMPs immediate early response genes (Suzuki et al., 1997). At least two different *Msx* genes, *Msx1* and *Msx2*, have been isolated from *Xenopus* (Su et al., 1991). Comparison of the expression pattern and especially the sequence strongly suggest that Msx1 and Msx2 may have similar functions during vertebrate development (Catron et al., 1996; Semenza et al., 1995). Experiments in chicken and mouse limb buds indicate *Msx-1* and *Msx-2* coexpress at many sites (Muneoka and Sassoon, 1992; Nohno et al., 1992). Moreover, the forced expression

of *Msx-2* mRNA in dorsal blastomeres induces a strong ventralized phenotype (Onitsuka et al., 2000), which is similar to *Bmp4* and *Msx1* (Maeda et al., 1997). However, some research revealed Msx2 may have distinct expression patterns and functions to Msx1. Results in *Xenopus* suggest that *Msx1* and *Msx2* have the same or similar expression in gastrula stages, but have different expression after the neurula stage. *Msx1* is expressed in broad areas, while *Msx2* is expressed in a narrower region of neural tissues (Onitsuka et al., 2000). Similar to *Xenopus*, findings in mouse development also indicated *Msx2* has more restricted expression pattern (Phippard et al., 1996). Although there are some differences between *Msx1* and *Msx2*, both of them can be regulated by BMP4 (Onitsuka et al., 2000; Suzuki et al., 1997). Combining these findings suggest it is possible that *Msx2* could be involved in BMP4 trigged superficial cell differentiation.

Another issue that has not been resolved is if Msx1 (or Msx2) or Ap-2 or Vent2 can activate Grhl expression. This could be investigated by overexpression of *Msx1*/*Ap-2*/*Vent2* RNA, followed by analysis of *Grhl1/3* expression.

4.3.3.2 Grhl and ESR6e

BMP4 regulates epidermal cell fate by activating transcription factors, including members of the Grhl family, which induce epidermal structure genes, such as *Keratin* (Onichtchouk et al., 1996; Suzuki et al., 1997). Grhl1 and Grhl3 share a high degree of identity in the DNA binding and dimerization domains (Ting et al., 2003). *Grhl1* and *Grhl3* also have similar ectodermally restricted patterns of expression (Ting et al., 2003). These data suggest that Grhl1 and Grhl3 may function redundantly in promoting superficial cell differentiation.

Grhl3 mutant mice are defective in re-epithelialisation following wounding (Ting et al., 2005). The expression of many tight-junction-associated proteins, such as occludins and claudins, was changed in Grhl knockout mice (Yu et al., 2006) and Grhl might directly regulate junction proteins (Narasimha et al., 2008). Thus, it is interesting to investigate the Grhl direct targets in *Xenopus*. Blocking *Grhl* by MOs in *Xenopus*, then screening the genes expression changes with microarrays, would identify potential targets. Chromatin Immunoprecipitations could then be used to establish which targets are direct. For example, investigating what is directly targeted by Grhl, for example, Grhl and some TJs genes promotors.

ESR6e is a bHLH protein of the Enhancer-of-split/hairy/HES family that responds to Notch activation and its normal expression is restricted to the superficial layer of epidermal ectoderm (Deblandre et al., 1999). Overexpression of *ESR6e* suppresses neuronal differentiation (Chalmers et al., 2002). This indicates that it may be another important molecule involved in superficial cells differentiation. The possibility could be proved by the same strategy used in this report: overexpression of *ESR6e* into deep cells, then screening superficial markers to see if those markers are expressed in the deep layers.

4.3.4 Gene X: a BMP4 inhibitor in the deep cells?

BMP4 (AC personal communication), Smad1/5/8 (Fig 4.11), *Msx1*, *Ap2* and *Vent* are all expressed in both layers. However BMP signalling activates Grhl1 and Grhl3 only in the superficial cells. It is tempting to speculate that an inhibitor(s) of differentiation maybe expressed by the deep cells. The inhibitor would stop activation of Grhl1 and Grhl3 in the deep cells, thus restrict differentiation to the superficial cells. The

mechanism provides a way to maintain undifferentiated stem/progenitor cells. Here, the inhibitor(s) is designated as gene X.

CtBP, C-terminal binding protein, is a known co-repressor of Smad-interacting protein-1 (Sip1) and inhibits BMP signaling (van Grunsven et al., 2007). In addition, CtBP can physically interact with Smad6 in BMP-induced Id1 transcription and negatively regulates BMP signaling (Lin et al, 2003). Consistent with the hypothesis it could be gene X, CtBP is strongly expressed in the epidermal deep cells (Fig. 4.11; van Grunsven et al., 2007). Vestigial-like 4, is also expressesed in deep layers (Chalmers et al, 2004; Fig 4.11). These results suggest that CtBP and possibly Vestigial-like 4 are good candidates for gene X.

There are other candidates for gene X. In *Xenopus* embryos, ectodermin is an inhibitor of Smad4 in both TGF-β and BMP pathways (Dupont et al., 2005). It is crucial for the ectoderm specification by inhibiting nodal and BMP (Dupont et al., 2005). The P53 homolog P63 is necessary to inhibit terminal differentiation of the epithelial layer by maintaining keratinocytes proliferative potency (Koster et al., 2004). Similar to P63, the putative functional homologue of mammalian Oct-3/4 in *Xenopus*, Oct-25 prevents the activation of genes required for terminal differentiation of tissues (Cao et al., 2008). Although it is not known if these genes are enriched in the deep cells, they are possible candidates of gene X. Analysing the normal expression of these genes in superficial and deep cells and overexpressing these genes in the superficial layers and investigating superficial markers expression, could be used to begin to test if these candidates are gene X.

4.3.5 Activation of deep gene expression

Deep genes such as hyaluronan synthase I and prothymosin alpha, are not activated by BMP4, suggesting that other signaling pathways promote expression of these genes. PAR1 (Partitioning-defective 1) is a possible candidate to activate these genes (Ossipova et al., 2007). PAR1 synergizes with XDelta-1 to induce ciliated cell differentiation and inhibits the superficial layer marker *ESR6e* expression. It was concluded that PAR1 specifies inner cell fates by inhibiting Notch signaling in the superficial ectoderm layer (Ossipova et al., 2007). The research group also demonstrated that PAR1 is downregulated by aPKC (Ossipova et al., 2007). Although aPKC is not sufficient to promote superficial differentiation, it may contribute to the superficial specification by inhibiting PAR1 function in superficial cells, so that deep genes can be promoted in the deep layers. It is possible PAR1 promotes gene X as well or it could even be gene X. The possibility that PAR1 can promote deep genes could be tested by blocking PAR1 with MOs in deep cells, and then checking deep gene expression.

4.3.6 Deep cells lack of polarity

Two polarity markers, aPKC (c-20) and ZO-1, were examed in the report. Overexpression of BMP4 attenuated aPKC apical localization and it is still unclear what inhibits the native aPKC localization. BMP4 did not establish cell junctions, marked by ZO-1, in the deep cells. Why was BMP4 not sufficient to assemble tight junction proteins and polarity molecules in *Xenopus* deep cells? It is possible that other signaling pathways contribute to the post-transcriptional regulation, including protein delivery and protein assembly. Exposure to an external environment may be

the key. To understand this issue more polarity and tight junction markers need to be examined and the possibility of other pathways participating also needs to be investigated.

4.3.7 Interactions between BMP and other pathways

In addition to BMPs, other signaling molecules such as WNTs, FGFs and Notch are involved in neural/epithelial specification, which may crosstalk with BMP4 and participate in the superficial differentiation. In *Xenopus*, WNT or activated β-catenin promotes the expansion of the neural crest (Chang et al., 1998), and Msx1 acts upstream of Xwnt-8 in the ventralizing signal cascade (Takeda et al., 2000). BMP-dependent activation of *Msx2* is mediated via a molecule downstream of WNT/β-catenin, lymphoid enhancer binding factor 1 (LEF1), and Smad4 (Hussein, et al, 2003). The two molecules synergistically bind and activate *Msx2* promoter (Hussein, et al, 2003). Those results suggest the possibility that WNT participates in superficial differentiation. Overexpressing WNT into *Xenopus* deep cells and screening for superficial markers expression could be used to test WNT function in the superficial differentiation.

In *Drosophila*, the *Grh* family is the target of many signaling pathways, including Notch (Lee and Adler, 2004). *ESR6e* is an ectodermal target of BMP4 signaling (Tao et al., 2005) and also responds to Notch activation (Chalmers et al., 2002). Ectopic expression of *Grhl1* induces *ESR6e* expression (Tao et al., 2005). These results strongly indicate that Notch should, like BMP4, promote superficial differentiation.

However, overexpression of Notch-ICD was not sufficient to promote superficial cell differentiation (see Chapter 3). The recently work on X-Tsukushi (X-TSK) might shed light on the question (Kuriyama et al., 2006). It was proposed that X-TSK plays a key role in the precise regulation of the neural and the non-neural ectoderm. X-TSK inhibits BMP by direct binding to BMP4 and via activation of Notch signaling (Kuriyama et al., 2006). Although there is no direct evidence about a feedback-loop between X-TSK and Notch, overexpressing *Notch-ICD* may promote X-TSK to inhibit BMP4. The existence of X-TSK may be an important reason why Notch-ICD can not promote the superficial cell differentiation. Knockdown of *X-TSK* by MOs when overexpressing *Notch-ICD* could be used to investigate if *Notch-ICD* is then able to promote superficial cell fate.

4.4 Summary

In this chapter, the role of BMP4 in promoting superficial cell differentiation in *Xenopus* was demonstrated. Via Msx1 or others molecules, BMP4 promotes the activation of superficial specific transcription factors, including Grhl1 and Grhl3. Those transcription factors promote the expression of epithelial structural proteins in the superficial cells. In contrast, BMP4 is not required for expression of deep genes. The mechanism which activates deep gene expression and blocks differentiation in deep cells remains unknown. However, a possibility is that an inhibitor, Gene X, is expressed by deep cells and blocks the BMP4 pathway downstream of Msx1. The next question that will be addressed is whether BMP acts to promote differentiation of the first epithelium in other vertebrates via a similar pathway.

5 Chapter V BMP4 can promote trophoblast cell fate in mouse embryonic stem cells

5.1 Introduction

Based on the previous investigations in *Xenopus* (Chapter IV), it was concluded that BMP4 initiates a transcriptional cascade via *Msx* and *Grhl* to promote differentiation of the first epithelium. The outside tissue of mouse embryos and *Xenopus* superficial layer are both made up of polarized epithelial cells. How similar are the mouse trophoblast and *Xenopus* superficial cells? Is their differentiation controlled by the same pathway? In this chapter, the effects of BMP4 treatment on mouse ES cells will be investigated to address the question of whether BMP4 promotes trophoblast formation.

5.1.1 BMP4 and the differentiation of stem cells

Accumulated evidence indicates that BMPs play important roles in stem cell biology. In *Drosophila* germline stem cells (GSCs), BMP2/4 is critical in stem cells maintenance (Xie and Spradling, 1998). In intestinal stem cells, BMP inhibits stem cells activation and expansion (He et al., 2004). In hematopoietic stem cells (HSCs), BMPs are essential for HSCs self-renewal (Zhang et al., 2003).

BMP4 treatment can promote epidermal formation in model organisms such as *Xenopus* (Wilson and Hemmati-Brivanlou, 1995). Recently it has been reported that mESCs can be induced to form epidermal cells following treatment with BMP4, which is similar to that in *Xenopus* (Medawar et al., 2008). They treated mESCs with

BMP4 from the third day to the fifth day, in the presence of serum, and found epidermal cells were included by BMP4 (Medawar et al., 2008). The findings are consistent with the research in other system, which indicates that BMP4 may function in a conserved manner in epidermal differentiation.

5.1.2 BMP4 and self renewal in mESCs

BMP4 also has a role in promoting self-renewal and maintaining mESCs pluripotency (Ying, et al., 2003a). A chemically defined serum-free culture media (N2B27) made it possible to investigate the role of single growth factors in mESC self-renewal/ differentiation (Ying et al., 2003b). By using the N2B27 media, a key experiment revealed that BMP4 acts synergistically with LIF to maintain mESC pluripotency (Fig 7) (Ying et al., 2003a). Withdrawal of BMP4 and LIF and culture in N2B27 media only, caused mESCs to form Sox1-positive cells and differentiate to neural lineage (Fig. 7).

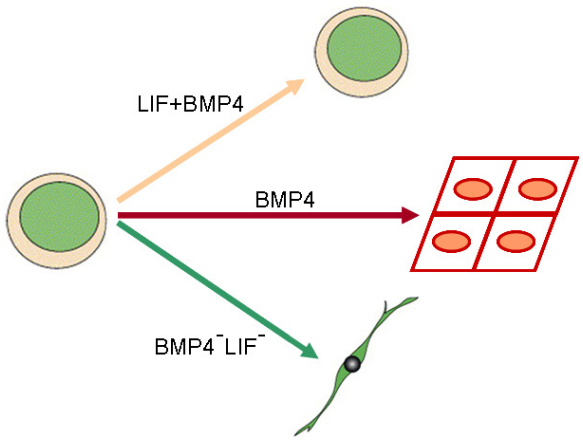


Fig 7 BMP4 acts synergistically with LIF to maintain mESC pluripotency. Adapted from (Ying et al., 2003b).

The research group explored the mechanism of the synergy between BMP4 and LIF.

They found that BMP4 activates Smad1 and the key gene *Id*, which blocks the formation of neural lineages. On the other hand, LIF signal through STAT3 blocks mesoderm and endoderm differentiation (Ying et al., 2003a), in part by nanog physically interacting with Smads (Suzuki et al., 2006). Thus, BMP4-Id collaborates with LIF-STAT3 to repress the lineage commitment and maintain pluripotency (Ying et al., 2003a).

LIF is not the only factor to crosstalk with the BMP pathway in balancing mESCs pluripotency and differentiation. For example, Phosphatidylinosital-3 kinase (PI3k)/Akt (a serine/threonine kinase) signaling is another well investigated pathway that can crosstalk with BMP4. By inhibiting Erk and P38 MAP kinase activity, PI3K/Akt enhances mESCs self-renewal and the subsequent differentiation (Paling et al., 2004). However, a study of mESCs derived from BMP4 receptor knockdown *Bmpr1a*$^{-/-}$ mouse indicates that Bmpr1a is required to suppress Erk/P38 activity and mESCs can not be established from *Bmpr1a*$^{-/-}$ mouse blastocysts without inhibition of P38 kinase activity (Qi et al., 2004). Thus, Smad pathway activation and MAPK pathway inhibition is involved in BMP-mediated maintenance of mESC (Paling et al., 2004; Qi et al., 2004). FGF signaling inhibits Smad activation via the MEK/Erk pathway (Aubin et al., 2004). Interestingly, Erk/P38 activity can be inhibited by the BMP-TAK1 cascade (Goswami et al., 2001; Qi et al., 2004). So antagonism between BMP and Erk MAPK pathways is essential for mESC self-renewal and maintenance.

5.1.3 Trophectoderm formation in mESCs

mESCs are not an obvious model to study trophectoderm formation because

experiments in chimeric mouse suggest mESCs are incapable of differentiating to the trophectodem lineage because they do not contribute to placenta in chimeras (Beddington and Robertson 1989). However, mESCs do have the potency to form trophectoderm *in vitro*. After culturing mESC derived embryoid bodies with serum-containing media, spontaneous expression of the trophoblast marker cytokeratin endo-A (CK8) was observed in LIF-free conditions (Toumadje et al. 2003). More recently, it was reported that a type IV collagen culturing system produced a small subset of trophoblast-like cells (Schenke-Layland et al. 2007). In addition decreased *Sox2* (Masui et al. 2007) or overexpressed *Ras* (Lu et al. 2008), *Cdx2* (Niwa et al., 2005), *Gata3* (Ralston et al., 2010), *Tead4* (Nishioka et al., 2008) or *Eomes* (Strumpf et al., 2005) also promotes trophoblast formation. In addition, methylation-deficient mESCs differentiate into trophoblast cells (Ng et al., 2008). From these findings, mESCs do have the potency to differentiate into the trophoblast lineages under certain conditions. *Wnt3a* is also able to induce a transient induction of Cdx2 expression in mESCs (He et al. 2008). However, there is currently no extracellular signaling molecule which is known to be able to promote stable trophectoderm formation.

BMP4 is perhaps an unlikely candidate to promote trophoblast cell fate given the role of BMP4 in self-renewal and differentiation of other lineages. However, one interesting observation came from Ying's experiment on the synergy of BMP4 and LIF in maintaining mESCs pluripotency. They noticed that withdrawn of LIF and culture in BMP4 caused mESCs to differentiate to form an epithelial like cell type of unknown fate (Fig. 7) (Ying et al., 2003b). In the last chapter, it was demonstrated that the BMP4 pathway is required and necessary for the *Xenopus* superficial

epithelium differentiation. This chapter will address the possibility that BMP4 can promote the trophoblast differentiation in mESCs.

5.2 Results

The chemically defined media, N2B27, was used as a basal media to investigate BMP4's role in mESCs differentiation. By using this media, Ying found mESCs stably differentiate into neural cells without additional growth factors (Ying et al., 2003a). They also noticed that mESCs differentiated to form epithelial like cells when BMP4 was added into N2B27. In this chapter, the cells produced by BMP4 treatment were analyzed in detail.

5.2.1 BMP4 promotes mESCs to form flattened sheets of epithelial like cells

Sox1-GFP mESCs cell line was cultured for four days with three different chemically defined media, starting with the same initial cell density. These chemically defined media were: N2B27 media with both BMP4 and LIF (Fig 5.1 A, B); N2B27 media with BMP4 (Fig 5.1 E, F), and the third was N2B27 without additional supplements (Fig 5.1 C, D). In all conditions, colonies formed normally. After culture in BMP4, cells differentiated to produce sheets of epithelial like cells (Fig 5.1 F, red arrows). No epithelium-like sheets were observed after culture in BMP4+LIF (Fig 5.1 B). Epithelial-like cells also did not occur in the N2B27 dishes without growth factors (Fig 5.1 D). The results were repeatable in R63 mESCs cell line (data not shown). Those observations were consistent with the previous report (Ying et al., 2003a).

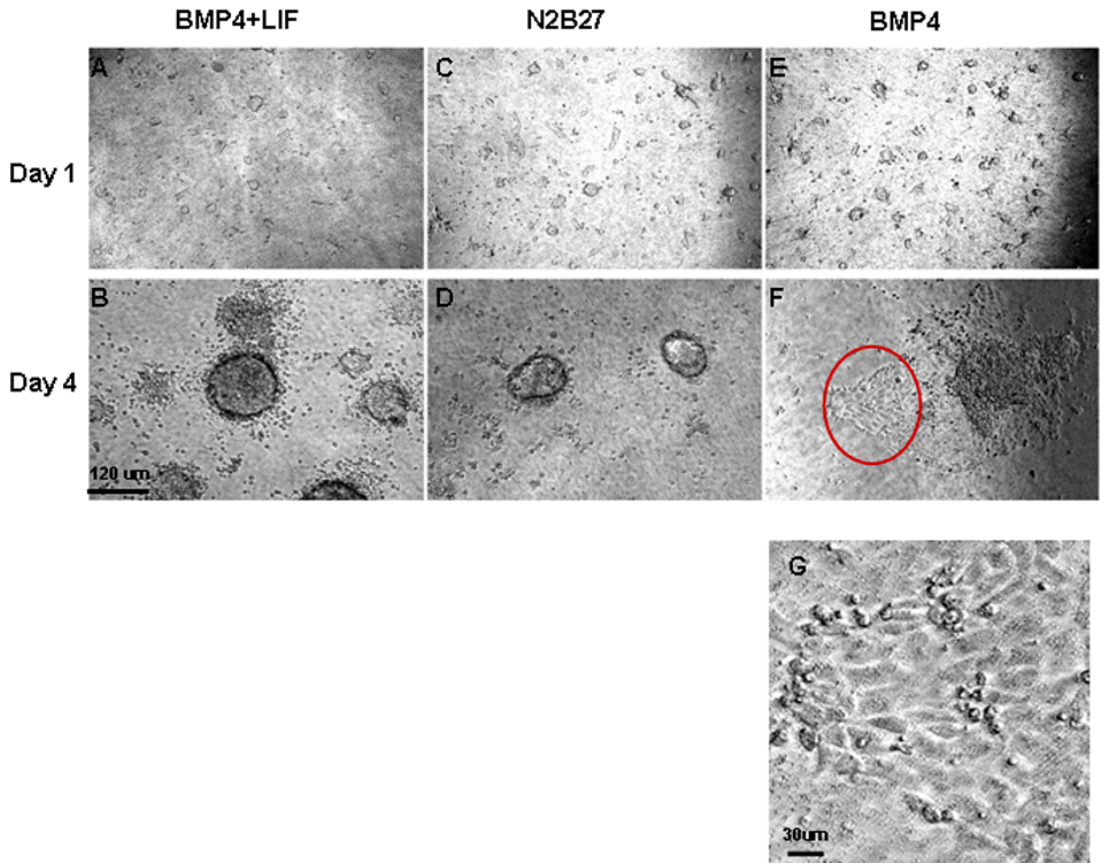


Fig 5.1 BMP4 promotes mESCs to form flattened sheets of epithelial like cells. (A+B) mESCs cultured in N2B27 media supplemented with BMP4 and LIF. (C+D) mESCs cultured in N2B27 media without growth factor supplement. (E-G) mESCs cultured in N2B27 media supplemented with BMP4 form flattened sheets of epithelial-like cells after 4 days of culture (red arrows). Based on at least 3 independent experiments.

5.2.2 BMP4 promoted junction protein formation in differentiated cells

To examine whether the flattened sheets induced by BMP4 are epithelial, the localization of junction proteins was examined by IF staining. mESCs (Sox1-GFP) were cultured in N2B27 media with BMP4 and LIF, N2B27 media with BMP4, and N2B27 only without any supplements. The localization of junction proteins, ZO1 and E-Cadherin, was investigated in the outer and inner part of a colony and the cells surrounding the colonies. The tight junction marker ZO1 was found in the membranes of the outer layer of cells of the colonies (Fig 5.2 G) and the surrounding cells in BMP4 treated dishes (Fig 5.2 I), but not in the cells inside the BMP treated colonies (Fig 5.2 H), in the BMP4+LIF treated cells (Fig 5.2 D-F), N2B27 media only cultured cells (Fig 5.2 A-C). .

E-Cadherin is a marker of adherens junctions. E-Cadherin was found in cells cultured in BMP4+LIF (Fig 5.2 M, N). After four days in culture some spontaneous differentiation occured around the edge of colonies, these cells had lost E-Cadherin staining (Fig. 5.2 O). E-Cadherin was found in the surrounding cells after BMP4 treatment (Fig 5.2 R). E-Cadherin was not observed in the colonies of BMP4 treated cells (Fig 5.2 P, Q), and N2B27 media only dishes (Fig 5.2 J-L). The localization of E-cadherin in pluripotent cells is consistent with previous work (Spencer et al., 2007), which indicates that E-cadherin is expressed in pluripotent mESCs and is lost during differentiation. The results from two types of junction proteins confirmed that BMP4 promotes epithelial polarization in mESCs. However, in the control BMP4+LIF cultures or N2B27 cultures epithelial polarization does not occur.

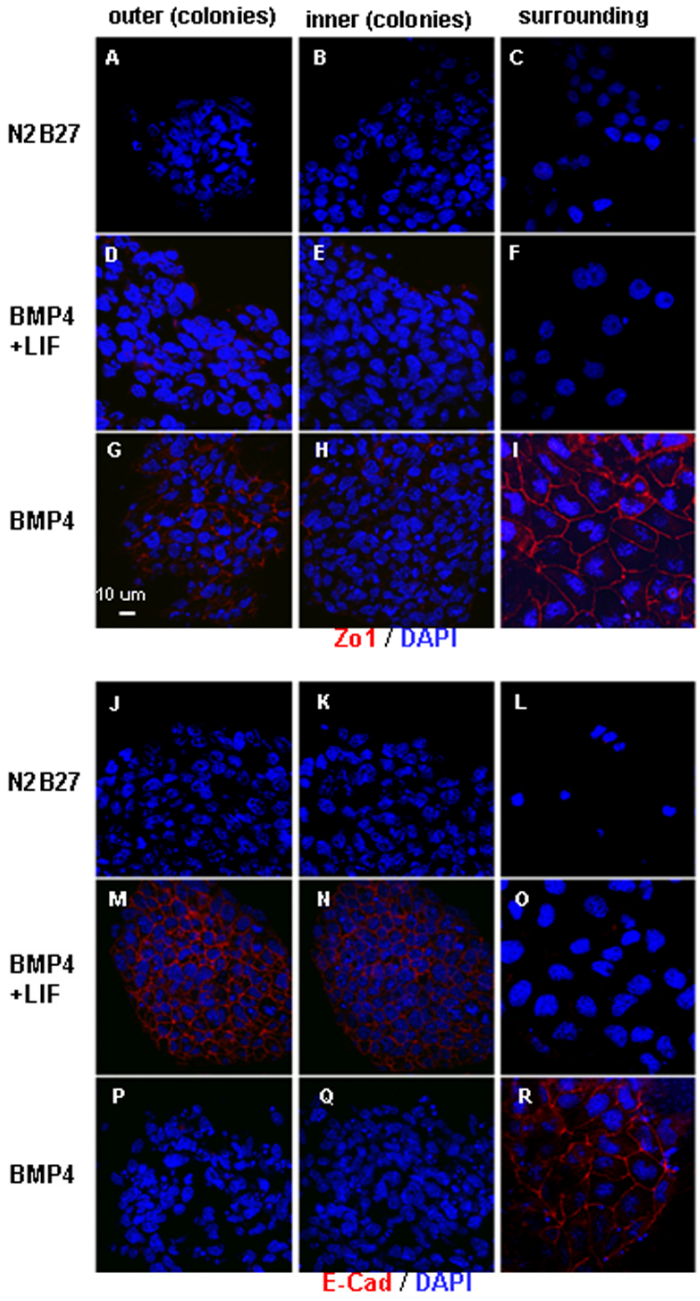


Fig 5.2 BMP4 promotes localisation of junction proteins in mESCs. (A+C) mESCs cultured in N2B27 media without growth factor supplement do not show membrane localized ZO-1. (D-F) mESCs cultured in N2B27 media supplemented with BMP4 and LIF do not show membrane localized ZO-1. (G-I) mESCs cultured in N2B27 media supplemented with BMP4 have membrane localized ZO-1 in the cells at the outside, but not inside, of colonies and in the cells which have spread from the colonies. (J-L) mESCs cultured in N2B27 media without growth factor supplement do not show membrane localized E-cadherin. (M-O) mESCs cultured in N2B27 media supplemented with BMP4 and LIF show membrane localized E-cadherin. (P-R) mESCs cultured in N2B27 media supplemented with BMP4 have membrane localized E-Cadherin in the cells which have spread from the colonies. For each treatment an image is shown of cells at the outside edge of a colony, of cells inside the colony and of cells which have spread away from the colony. Based on at least 3 independent experiments.

5.2.3 Culture in BMP4 causes a loss of pluripotency and a block on neural differentiation

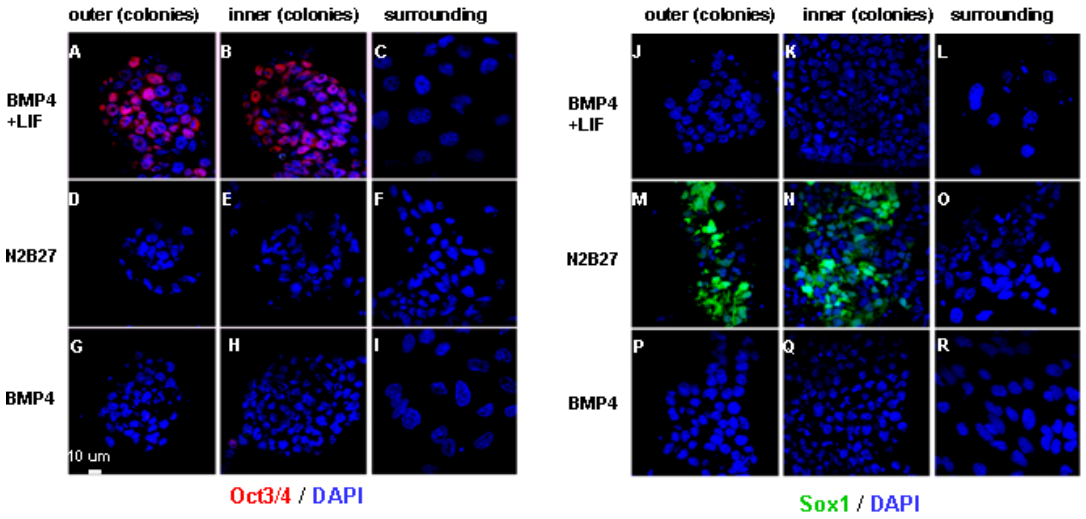
Are the BMP4 treated cells losing pluripotency? The issue was assessed by investigating the expression of Oct3/4 (Nichols et al., 1998). Oct3/4 expression was examed in the outer and inner part of a colony and the differentiated cells via IF staining. mESCs (Sox1-GFP) were treated with BMP4 plus LIF, BMP4, N2B27 media only respectively. Only colonies in the BMP4+LIF treatment dishes showed Oct3/4 positive cells (Fig 5.3 A, B). The other two treatments did not have Oct3/4 positive cells (Fig 5.3 D-I).

Sox1 is the marker for early neural development (Pevny et al., 1998) and neural differentiation was investigated by using Sox1-GFP expression. The cells cultured in N2B27 media alone developed Sox1-GFP positive cells (Fig 5.3 M, N), whereas colonies in other treatments (Fig 5.3 J-L, P-R) and the surrounding cells (Fig 5.3 O) did not express Sox1-GFP.

To confirm the result that culture in BMP4 causes a loss of pluripotency and blocks neural differentiation in mESCs, a number of pluripotency markers were investigated by RT-PCR after 5 days treatment. Compared to BMP4 + LIF treatment, pluripotency genes Nanog and Oct3/4 were found to be down-regulated after BMP4 treatment. Expression of the neural induction marker Sox1 was only observed in the N2B27 media only sample (Fig. 5.3 S).

Taken together, these two sections showed that BMP4 treatment alone results in a loss of pluripotency, and the formation of epithelial cells with characteristic adherens and

tight junctions. This indicates that BMP4 promotes epithelial differentiation.



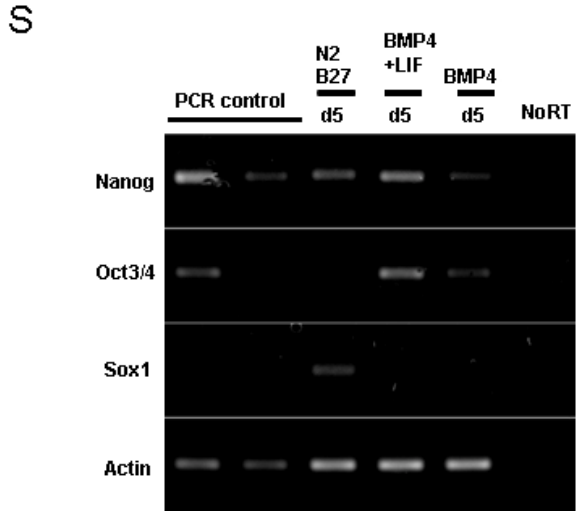


Fig 5.3 mESCs cultured in BMP do not express pluripotency or neural markers. (A+C) Expression of Oct3/4 in mESCs cultured in N2B27 media supplemented with BMP4 and LIF. (D-F) Expression of Oct3/4 in mESCs cultured in N2B27 media without growth factor supplement. (G-I) Expression of Oct3/4 in mESCs cultured in N2B27 media supplemented with BMP4. (J-L) Expression of Sox1-GFP in mESCs cultured in N2B27 media supplemented with BMP4 and LIF. (M-O) Expression of Sox1-GFP in mESCs cultured in N2B27 media without growth factor supplement. (P-R) Expression of Sox1-GFP in mESCs cultured in N2B27 media supplemented with BMP4. For each treatment an image is shown of cells at the outside edge of a colony, of cells inside the colony and of cells which have spread away from the colony. (S) RT-PCR analysis confirmed that after 5 days of culture BMP4 treated cells express low levels of the pluripotency markers *Nanog* and *Oct3/4* and the neural marker *Sox1*. A mixture of mESC samples from day 1 to day 5 RNA was used as the PCR control. Based on at least 3 independent experiments.

5.2.4 BMP4 promotes trophoblast cell fate

What is the fate of the epithelial cells induced by BMP4 treatment, there are three main possibilities: mESCs can spontaneously form primitive endoderm in embryoid bodies (Hamazaki et al., 2004), suggesting it could be primitive endoderm. BMP4 can also promote epidermal cell fate (Medawar et al., 2008). Finally results from *Xenopus* (Chapter 4) suggest it could be trophectoderm.

To answer the question, mESCs (Sox1-GFP) were cultured in N2B27 media with BMP4 and BMP4 plus LIF respectively. Two trophectoderm markers, Cdx2 (Beck et al., 1995) and Troma1 (Brulet et al., 1980), and the primitive endoderm marker Hnf4α (Hamazaki et al., 2004) were analyzed by IF. In BMP4 dishes (Fig 5.4), after 4-5 days culture, Cdx2 (Fig 5.4 A-C') and Troma1 (Fig 5.4 D-F') were strongly expressed in the outer cells of the colonies and the surrounding cells but less in inner cells. BMP4 treatment did not promote HNF4α (Fig 5.4 G-I') expression. The percentage of positive cells for each marker in the cells surrounding the colonies was quantitated as described in the Materials and Methods. The mean of at least three independent experiments, +/- the standard deviation, is shown graphically (Fig 5.4 J and in Table 8). BMP4 treatment produced a high percentage of the surrounding cells which were with positive for Cdx2 (88.19%) and Troma1 (96.44%), but very low percentage for HNF4α positive (8.01%).

Cdx2 (Fig 5.5 A-C') and Troma1 (Fig 5.5 D-F') expression was not widely observed after BMP4 and LIF treatment. However, in BMP4 and LIF treatment dishes (Fig 5.5), some spontaneous differentiation occurred around the edge of the colonies, in these cells HNF4α (Fig 5.5 G-I') was strongly expressed. The percentage of

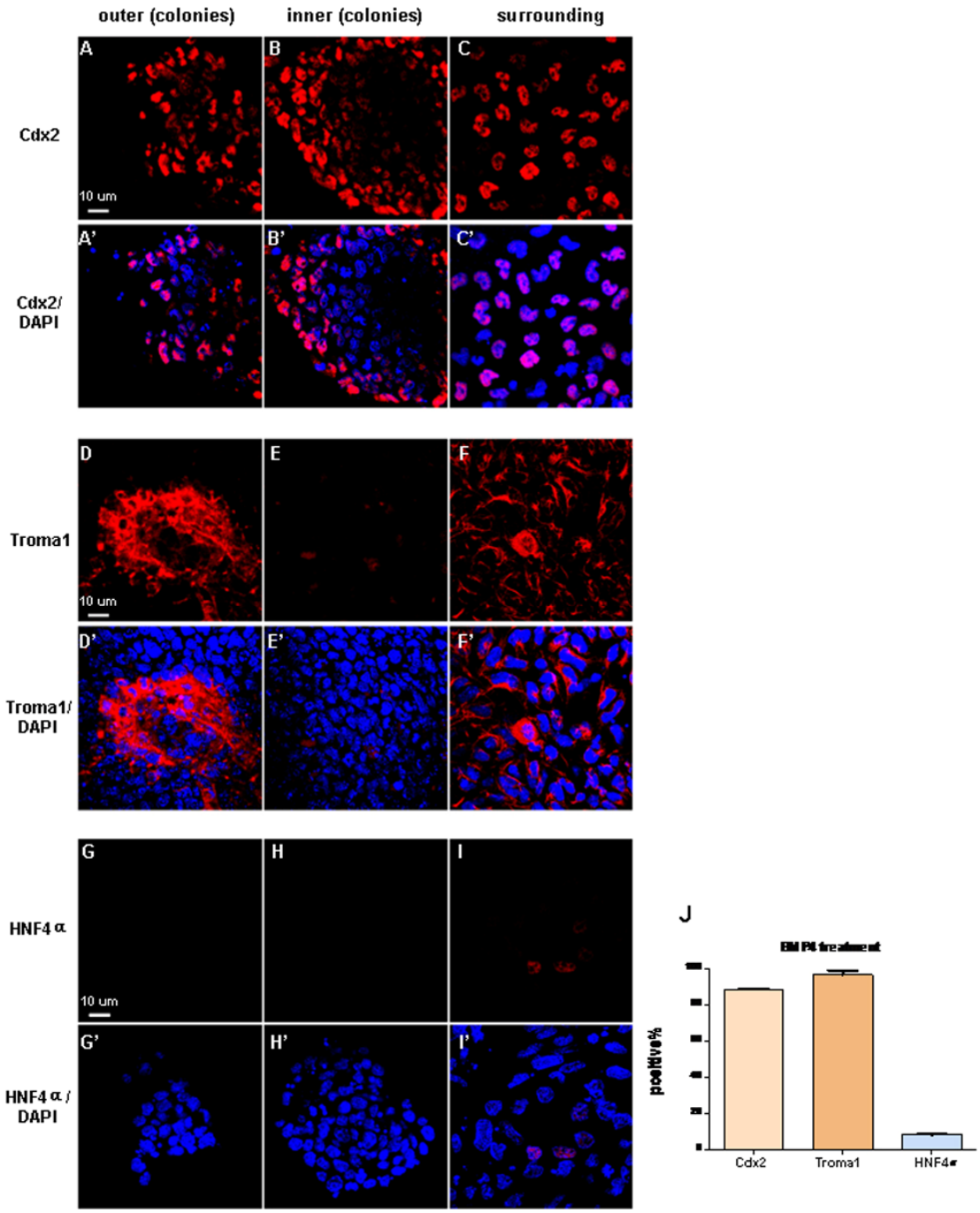


Fig 5.4 BMP4 induces mESCs to expresses markers of trophectoderm. (A+C) A high percentage of cells express Cdx2 after culture in N2B27 media with BMP4. (D-F) A high percentage of cells express Troma1 after culture in N2B27 media with BMP4. (G-I) A low percentage of cells expression HNF4α after culture in N2B27 media with BMP4. For each antibody an image is shown of cells at the outside edge of a colony, of cells inside the colony and of cells which have spread away from the colony. (J) Quantification of the fate of the cells which surround the colonies. The mean percentage of cells positive for each marker, +/- the standard deviation, is presented. Calculated from at least three independent experiments. mESCs were cultured for four days.

positive cells for each marker in the surrounding cells was quantitated as described in the Materials and Methods. The mean of at least three independent experiments, +/- the standard deviation, is shown graphically (Fig 5.5 J) and in Table 8. After culture in BMP4 and LIF a high percentage of the surrounding cells were positive for HNF4α (91.61%), but low for Cdx2 (9.54%) and Troma1 (19.56%).

Cdx2 (Fig 5.6 A-C') and Troma1 (Fig 5.6 D-F') expression are not widely observed after culture in only N2B27. However, HNF4α (Fig 5.6 G-I') was expressed in some of the surrounding cells. The percentage of positive cells for each marker in the surrounding cells was quantitated as described in the Materials and Methods. The mean of at least three independent experiments, +/- the standard deviation, is shown graphically (Fig 5.6 J and Table 8). Culture in N2B27 media produced a low percentage of surrounding cells positive for Cdx2 (13.53%), Troma1 (13.71%) and HNF4α (31.07%).

In summary this data suggests that culture in BMP4 mainly promotes trophoblast cell fate, while spontaneous differentiation produces mainly primitive endoderm formation.

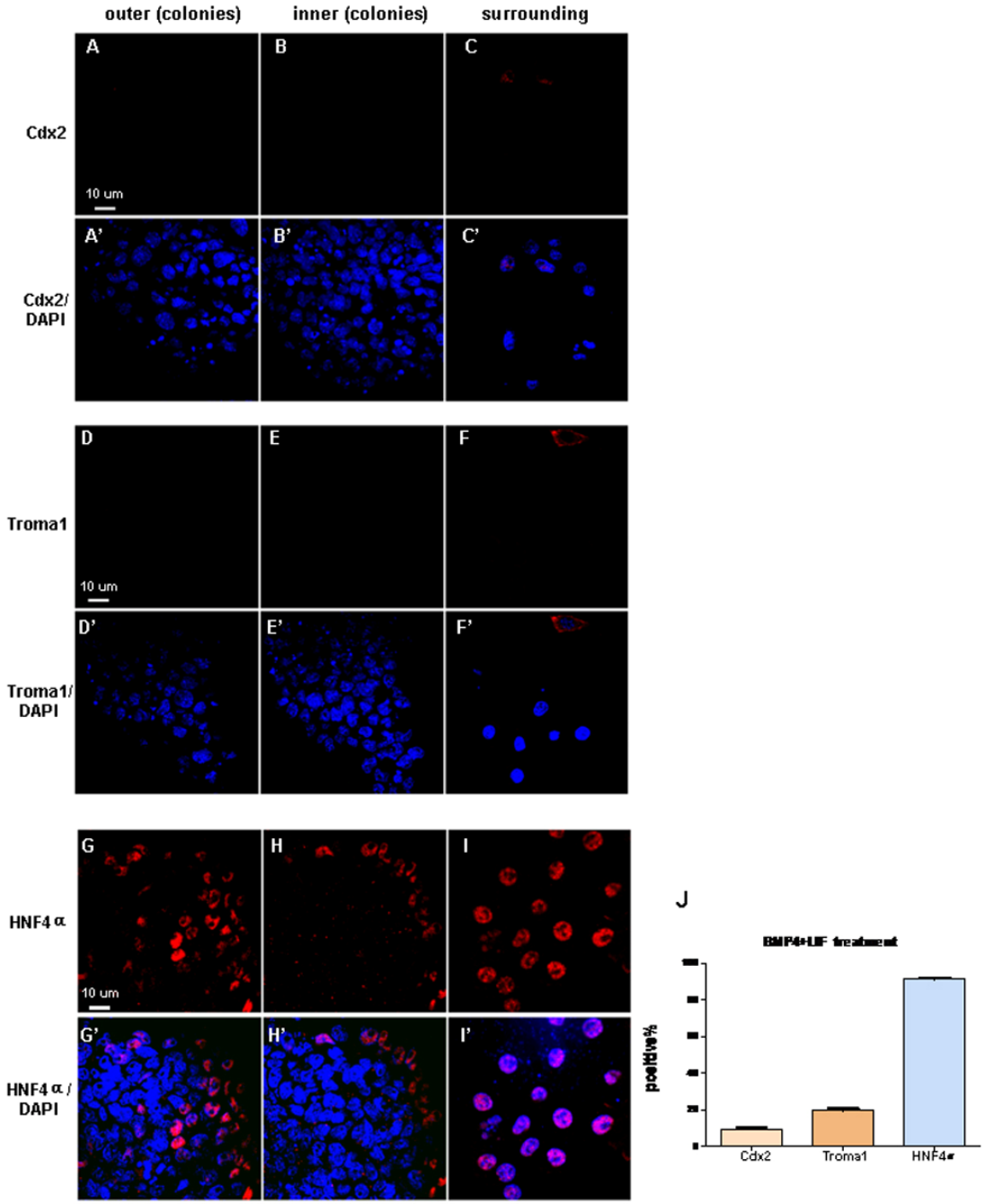


Fig 5.5 Some mESCs cultured in BMP4+LIF expresses markers of primitive endoderm. (A+C) A low percentage of cells express Cdx2 after culture in N2B27 media with BMP4 + LIF. (D-F) A low percentage of cells express Troma1 after culture in N2B27 media with BMP4 + LIF. (G-I) A high percentage of cells express HNF4α after culture in N2B27 media with BMP4 + LIF. For each antibody an image is shown of cells at the outside edge of a colony, of cells inside the colony and of cells which have spread away from the colony and differentiated. (J) Quantification of the fate of the cells which surround the colonies. The mean percentage of cells positive for each marker, +/- the standard deviation, is presented. Calculated from at least three independent experiments. mESCs were cultured for four days.

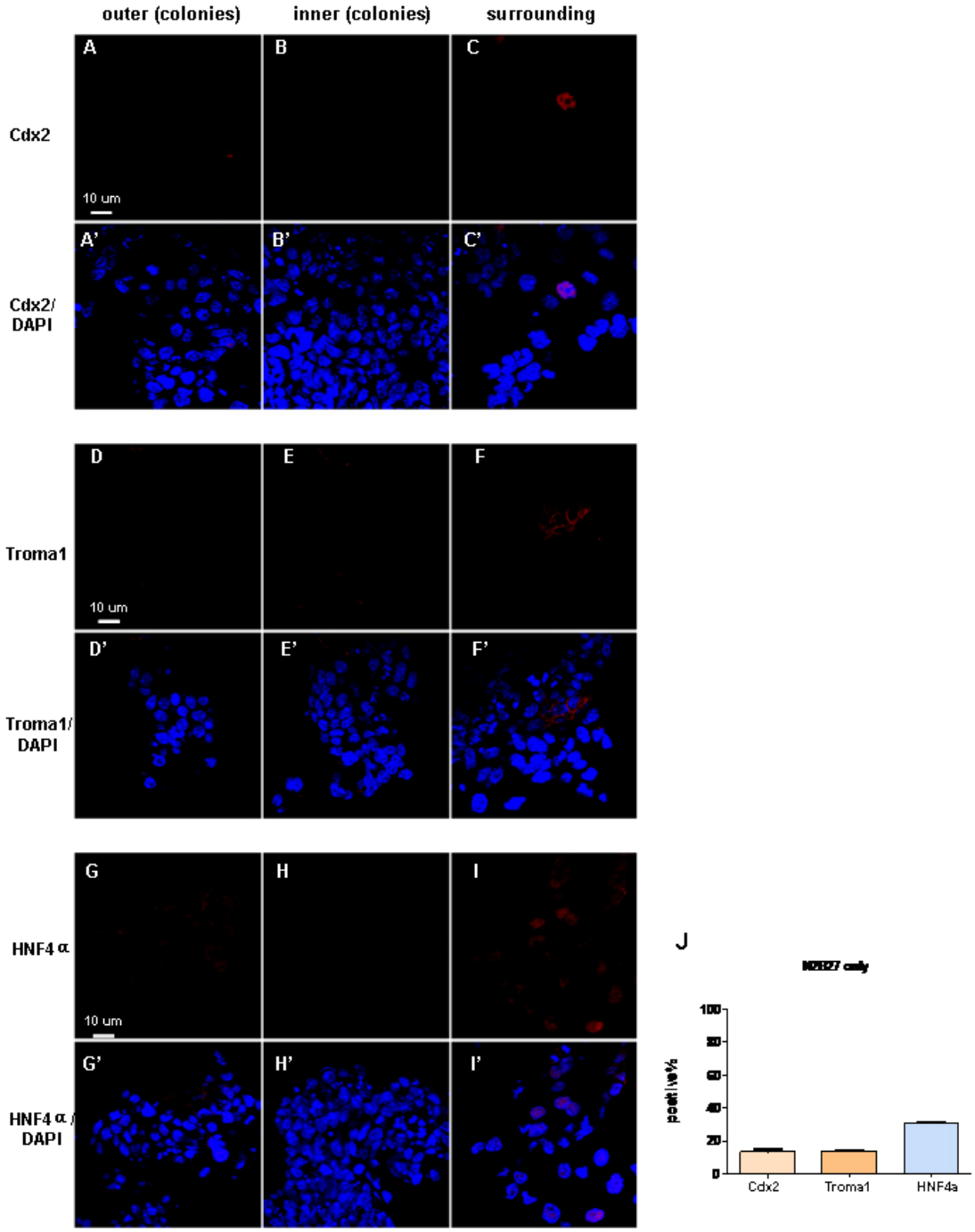


Fig 5.6 A low percentage of mESCs cultured in N2B27 express markers of trophectoderm or primitive endoderm. (A+C) A low percentage of cells express Cdx2 after culture in N2B27 media. (D-F) A low percentage of cells express Troma1 after culture in N2B27 media. (G-I) A low percentage of cells express HNF4α after culture in N2B27 media. For each antibody an image is shown of cells at the outside edge of a colony, of cells inside the colony and of cells which surround the colony. (J) Quantification of the fate of the cells which surround the colonies. The mean percentage of cells positive for each marker, +/- the standard deviation, is presented. Calculated from at least three independent experiments. mESCs were cultured for four days.

Table 8 BMP4 contribute to TE markers expression

staining		No.of positive	No. of negative	No. of all differentiated cells	% of every experiment	average of %	standard deviation	P Value (BMP4+LIF VS BMP4)
Cdx2	BMP4 +LIF	9	84	93	9.68	9.544	0.01334	2.56264E-11
		11	88	99	11.10			
		8	71	79	10.10			
		5	64	69	7.46			
		6	58	64	9.38			
	N2B27	10	54	64	15.62	13.53	0.02710	
		28	165	193	14.51			
		11	94	105	10.47			
	BMP4	288	38	326	88.34	88.19	0.00607	
		112	16	128	87.50			
		177	22	199	88.94			
		205	28	233	87.98			
Troma1	BMP4 +LIF	30	110	140	21.42	19.56	0.018121	8.18E-06
		31	143	174	17.80			
		29	120	149	19.46			
	N2B27	19	135	154	12.33	13.71	0.01262	
		12	69	81	14.81			
		13	80	93	13.98			
	BMP4	155	13	169	91.72	96.44	0.041838	
		314	1	315	99.70			
		186	4	190	97.89			
HNF4α	BMP4+ LIF	244	20	264	91.73	91.61	0.013887	7.50271E-08
		79	6	85	92.94			
		266	29	295	90.17			
	N2B27	66	144	210	31.43	31.07	0.00315	
		37	83	120	30.83			
		61	136	197	30.96			

	BMP4	16	169	185	8.64	8.01	0.00645	
		15	189	204	7.35		8	
		19	117	236	8.05			

To further confirm the induction of trophoblast cell fate, a number of markers were investigated by RT-PCR after 5 days treatment. Primitive endoderm genes, *Hnf4a* and *Gata4* (Arceci et al., 1993), were lower after BMP4 treatment. These results are consistent with the IF results (Fig 5.7 A) and suggest that BMP4 treatment does not promote primitive endoderm formation in mESCs. Since data from other model organisms suggests that BMP treatment of naïve ectoderm promotes surface epidermis (Knecht and Bronner-Fraser, 2002; Ster CD., 2001), the epidermal marker *p63* (Laurikkala et al., 2006) was also investigated. Although expressed by embryoid bodies at day 6, *p63* was not detected in mESCs after the three treatments. These results argue that in this system BMP4 is not sufficient to promote epidermal formation.

Markers of the trophectoderm lineage were analysed. The group included markers of early trophectoderm, *Cdx2*, *Eomes* (Ciruna and Rossant, 1999), *Psx1, Dlx3, Fgfr2* (Niwa et al., 2000); ecto-placental cone markers *Mash2, Tpbp* and *Psg19* (Hayashi et al., 2010); trophoblast giant cells placental lactogen-2 (*PL-2*) (Faria et al., 1991). Those markers were all higher or specifically expressed in cells cultured in BMP4 at the fifth day (Fig. 5.7 B), which confirms that BMP4 induces trophoblast differentiation in mESCs.

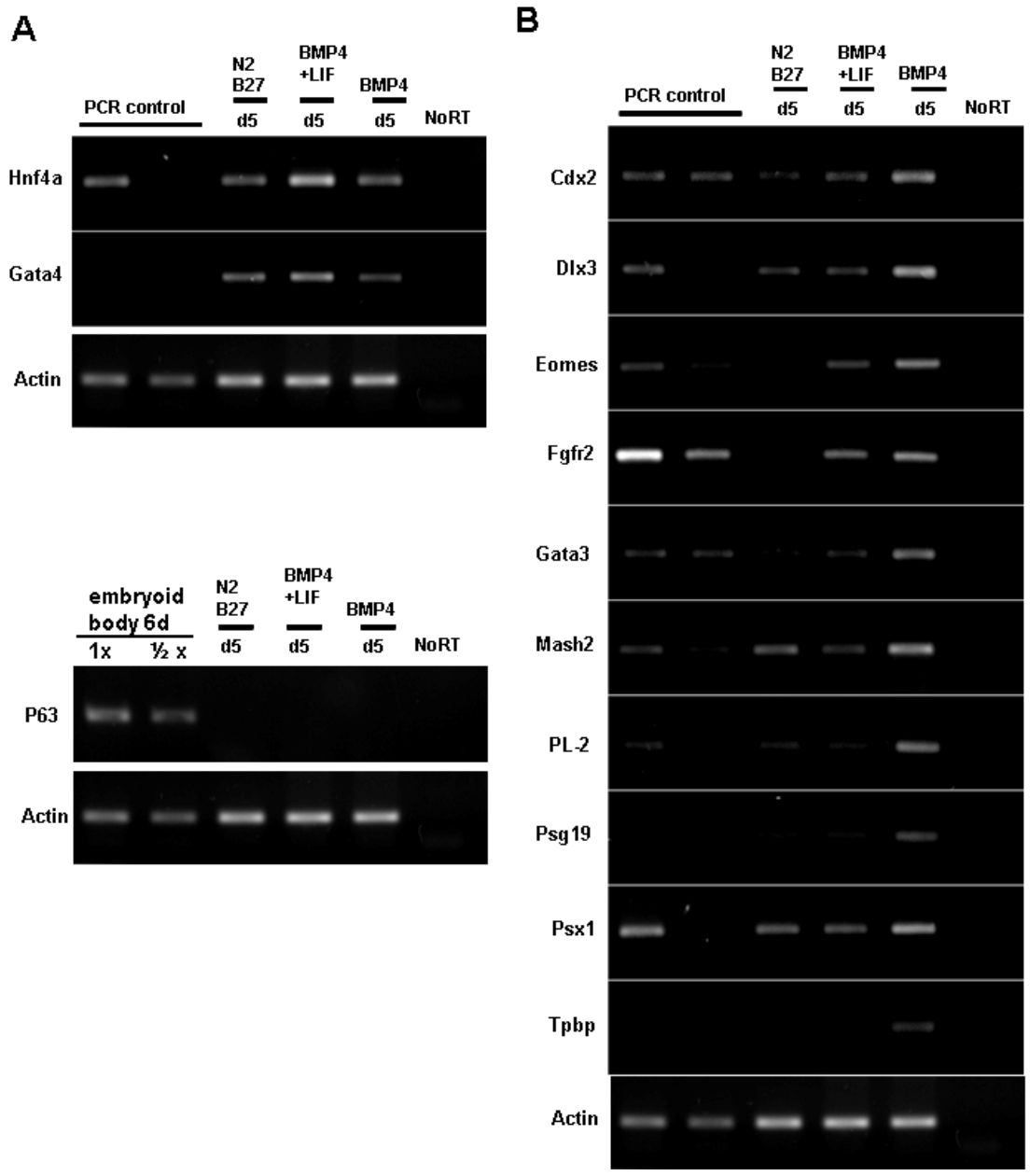


Fig5.7 BMP4 promoted expression of a range of trophectoderm genes. (A) ES cells cultured in BMP4 express low levels of primitive endoderm markers. (B) ES cells cultured in BMP4 express high levels of trophoblast markers. (C) Expression of *p63*, a surface ectoderm marker, was not detected in ES cells cultured with BMP4. The RT-PCR analysis was carried out after 4 days in culture. A mixture of mESC samples from day 1 to day 5 RNA was used as the PCR control, except for C which used 6 day old embryoid bodies. Based on at least 3 independent experiments.

It was also noticed that after BMP4 treatment, there were a few very large cells that were much bigger than other cells (Fig 5.8 C; red circles shown). No similar cell type was observed in other two treatments (Fig 5.8 A, B). Trophoblast cells develop into polyploid trophoblast giant cells, which are much bigger in size. The large cells could be trophoblast giant cells. This hypothesis is supported by the expression of the giant cell marker *Pl-2* seen by RT-PCR.

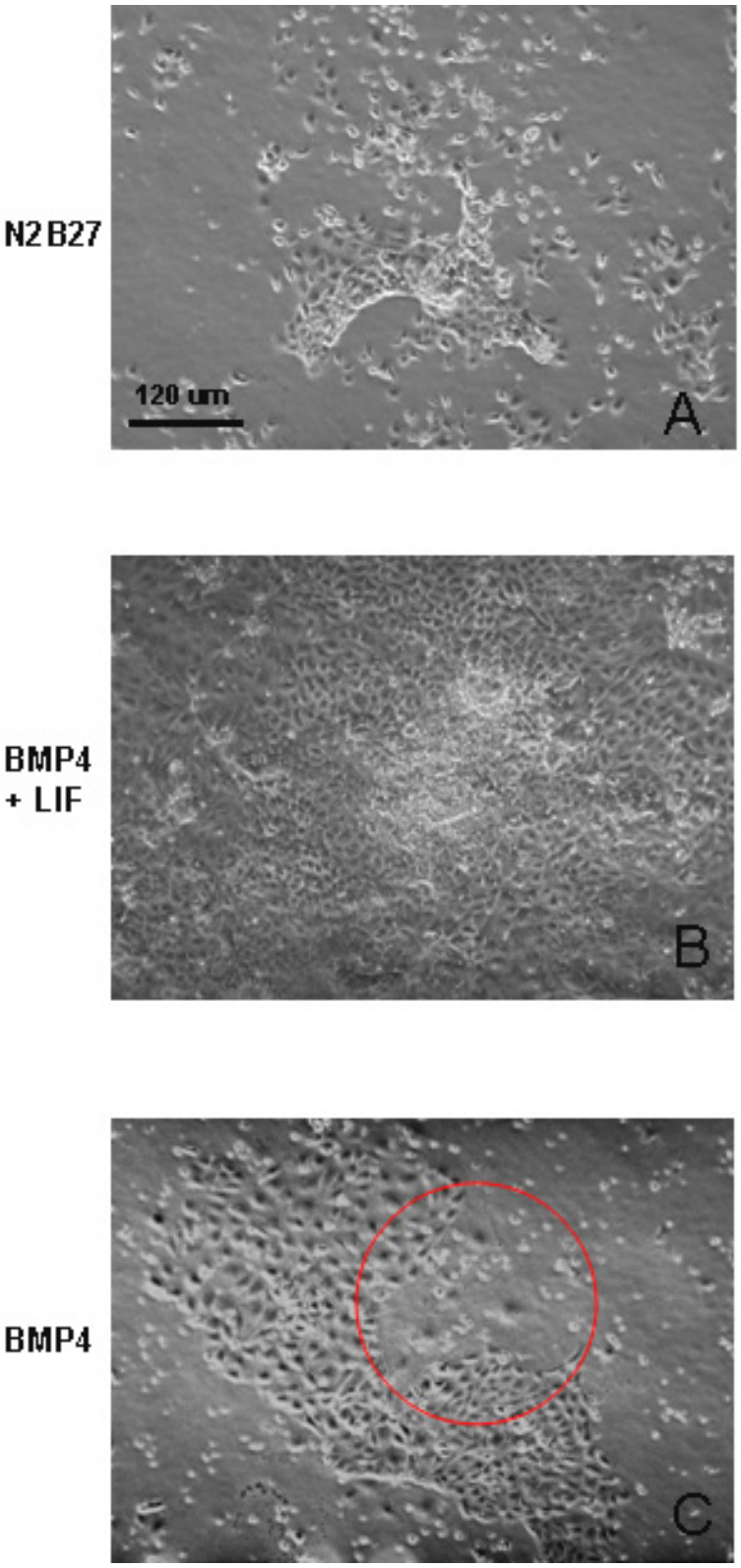


Fig 5.8 After culture in BMP4 some very large cells are formed. (A) mESCs cultured in N2B27 media without growth factor supplement. (B) mESCs cultured in N2B27 media supplemented with BMP4 and LIF. (C) mESCs cultured in N2B27 media supplemented with BMP4 form a few very large cells after 8 days of culture (red circle). These cells could be trophoblast giant cells. Based on at least 3 independent experiments.

5.2.5 BMP4 promotes expression of Msx and Grhl transcription factors

In *Xenupus*, BMP4 promotes differentiation of the first epithelium via the Msx and Grhl transcription factors. In mESCs, BMP4 also promotes the first epithelium, trophoblast formation. It is interesting to investigate if the same set of transcription factors were activated.

Grhl1, Grhl3, Msx1, Msx2, the transcription factors which are activated by BMP in *Xenopus* embryos, were tested in mESCs by RT-PCR. mESC cell line (Sox1-GFP) was cultured in N2B27 media with BMP4 plus LIF, N2B27media with BMP4, and N2B27 media only. RNAs were extracted from each group at the fifth day respectively. Expression of *Msx1*, *Msx2* and *Grhl1* are all enriched in BMP4 treated cells (Fig 5.9). These results are similar to those in *Xenopus*. Expression of *Grhl3* was detected after culture in BMP4+LIF, however the expression in BMP4 treatment appeared stronger. Thus, BMP4 activates a similar set of transcriptional regulators during induction of superficial cells in *Xenopus* and trophoblast differentiation in mouse ES cells.

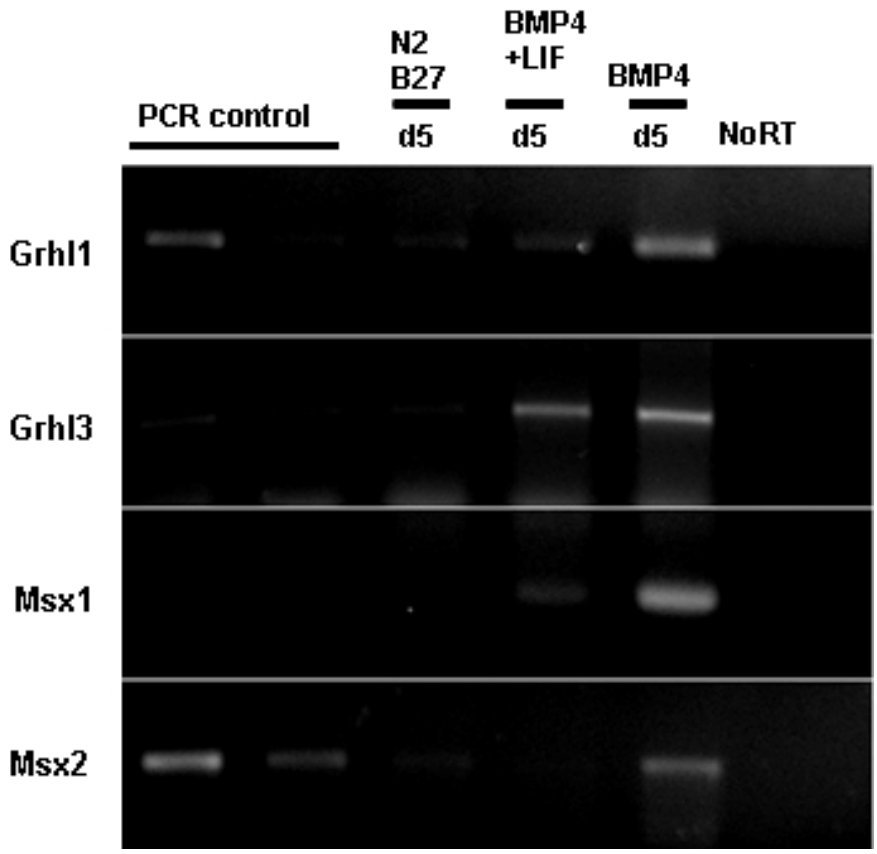


Fig5.9 Expression of Msx and Grhl transcription factors was promoted by BMP4 in ES cells. RT-PCR analysis of mouse ES cells after 4 days in culture shows that BMP4 treatment activates the expression of members of the Msx and Grhl families of transcription factors. A mixture of mESC samples from day 1 to day 5 RNA was used as the PCR control. Based on at least 3 independent experiments.

5.3 Discussion

5.3.1 BMP4 induced trophoblast cell differentiation

Previous work noticed that BMP4 treated mESCs cultured in N2B27 suppress neural differentiation and form sheets of large flat cells, however the identity of these BMP4-induced cells remained unknown. Ying‘s report indicated that the cells were not mesoderm (Ying et al., 2003a,b). Kunath and colleagues thought a minority of the cells might be immature ectoderm as a small subset expressed keratin 14 (Kunath et al., 2007). Previous research had not considered that the cells may be trophectoderm, perhaps because mESCs do not contribute to trophectoderm in chimeras. This study shows that mESCs cultured in BMP polarise and differentiate along the trophoblast lineage.

A recent independent study showed that when cocultured with laminin, BMP4 promotes trophectoderm differentiation in mESCs (Hayashi et al., 2010). However, the culture conditions used did not allow the authors to demonstrate which was driving trophoblast fate differentiation, the addition of BMP4, or removal of LIF. Another difference between their study and this project is the requirement of laminin for trophoblast induction: our culture system demonstrated laminin is not required for trophectoderm differentiation, while they think it is. In addition, our project suggests potential transcription factors that may be involved in trophoblast cell fate specification.

The tight junction localization and trophoblast markers shared the same spatial pattern (Fig 5.5), the outer cells in the colonies expressed the lineage markers while the cells

inside do not. The results suggest that cell polarization might affect the expression of the genes that specify trophoblast cell fate. This matches the current *in vivo* model that cell polarity establishment is a necessary factor for trophectoderm differentiation (reviewed by Johnson and McConnell, 2004). This suggests that BMP4 could be promoting cell polarity and then cell polarity is promoting trophoblast cell fate. Alternatively, BMP4 might be promoting cell polarization and cell fate by two independent pathways. A first step to investigate this issue would be to look the timing of polarization and expression of trophoblast genes.

5.3.2 Transcription factors down stream of BMP4

Our research proved that BMP4 activates trophoblast differentiation in mESCs. Moreover, Msx and Grhl are also activated during such the differentiation. Although a large number of trophectoderm lineage markers were up-regulated by BMP4, the relationship between Msx/Grhl and trophoblast specific genes were not established.

As Msx is a direct target of BMP4, it suggests that trophoblast gene expression may be at the end of the BMP4-promoted cascade, like *Keratin* or *Claudin* in *Xenopus*. Thus, Cdx2, an important TE lineage regulator, maybe be induced by Msx. However, Hayashi and co-worker (Hayashi et al., 2010) found when cultured with laminin, trophoblast formation was triggered by direct activation of *Cdx2* expression by Smad pathway. The discovery suggests an alternative possibility that *Cdx2* may not be activated by Msx. In addition, an FGF4-FRS2α (fibroblast growth factor receptor substrate 2α)-Cdx2 axis in trophoblast stem cells can induce *Bmp4* expression (Murohashi et al., 2010). It is known that ES cells produce fibroblast growth factor 4

(FGF4) (Nichols et al., 1998), which activates ERK (the extracellular signal-regulated kinase) signalling (Thisse and Thisse, 2005). In response to FGF4, FRS2α activates ERK pathway to enhance expression of Cdx2 in TS cells. Via binding to the promoter region of *Bmp4*, Cdx2 in turn leads to production and secretion of BMP4. Moreover, *Bmp4* can rescue *Frs2α*-null ICM from the defective growth (Murohashi et al., 2010). As discribed in Fig 8, this suggests there may be a feedback loop between trophoblast genes and BMP4 (Murohashi et al., 2010). The regulation of Msx1 by ERK (Bushdid et al., 2001) also suggests that the feedback loop may possibly involve Msx/Grhl.

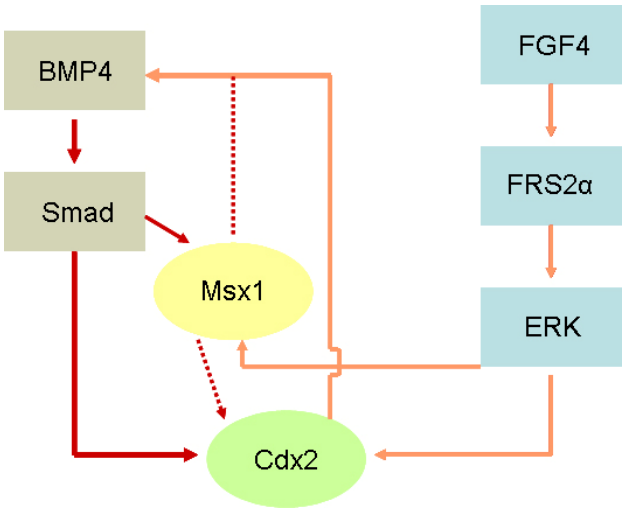


Fig 8 a feedback loop between trophectoderm marker Cdx2 (green), FGF4 (blue) and BMP4 (grey), Msx1(yellow) may play a centre role in the feedback loop. Arrows indicate confirmed positive interaction, while broken lines point the possible interaction need to be confirmed.

Cdx2 or Msx knock-down experiments are needed to establish if there are links between these transcription factors. A Msx1-null mESC line could be generated, and the cells cultured with BMP4 and then investigate TE formation by examining Cdx2 expression. If Cdx2 is not expressed, the result supports the idea that Cdx2 is the target of Msx1. If the number of Cdx2 positive cells does not change, it indicates that Msx1 is not a major regulator of Cdx2.

5.3.3 BMP signalling and other pathways involved in trophoblast differentiation

5.3.3.1 WNT3a and LEF

The WNT pathway is a candidate for promoting self-renewal because previous studies in human and mouse ES cells showed that activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor sustains pluripotency (Sato et al., 2004). However, *Wnt2* and *Wnt7b* knockouts show placenta defects (Parr et al., 2001), suggesting a role of Wnt signaling in the TE lineage development. Recent findings revealed that WNT signalling transiently induces Cdx2 expression in mESC (He et al., 2008). WNT signalling is known to stabilize BMP-Smad signals (Fuentealba et al., 2007). Based on our work, it seems possible that the transient induction of *Cdx2* caused by WNT signalling could be due to enhancement of endogenous BMP signalling by stabilizing Smad signaling.

Mice with Lef1/Tcf1 knockout can not form placenta properly, similar to the wnt3a mutant phenotype (Galceran et al., 1999). That is because LEF-1/TCF proteins associate with β-catenin and activate transcription (reviewed by Galceran et al., 2001). Recently, He's report revealed that Cdx2 can be induced in response to Wnt3a, which is mediated by LEF1 (He et al., 2008). BMP4 is also sufficient to promote LEF1 expression (Kratochwil et al., 1996). In contrast LIF, via STAT3, significantly represses LEF1 induction (Sachdev et al., 2001). These connections suggest there is crosstalk between BMP, WNT and LIF on LEF1 regulation. .

5.3.3.2 FGF and MAPK

Fibroblast growth factors (FGFs) activate the Ras-Erk signalling cascade and are critical for proliferation and differentiation in many cell types (Thisse and Thisse, 2005). Fgf4 is required for trophoblast stem cell maintenance and proliferation (Tanaka et al., 1998) and in mouse embryos Fgf4 is produced by the developing ICM cells in direct response to Oct4/Sox2 (Nichols et al., 1998). The essential role of the FGF4 pathway is confirmed in the defective trophoblast development and peri-implantation lethality of embryos lacking various pathway components (*Saba-El-Leil* et al., 2003; Gotoh et al., 2005). Fgf receptor 2 (Fgfr2), which is strongly expressed in the proliferating trophoblast, is a candidate receptor for receiving the FGF4 signal (Ornitz & Itoh, 2001).

Previous work looking at the epithelial like cell type produced by BMP4 used an *Fgf4*$^{-/-}$ ES cell line and an FGFR inhibitor (Kunath et al., 2007). Kunath's work demonstrated that FGF signalling pathway is essential for BMP induced differentiation. When this work was carried out, the identify of the cells induced by BMP4 treatment was unknown, but when combined with my study it suggests that BMP activates FGF, which then activates ERK and drives trophoblast cell fate specification. The fact that Fgfr2 expression was up-regulated by BMP4 (this work) partially supports this hypothesis.

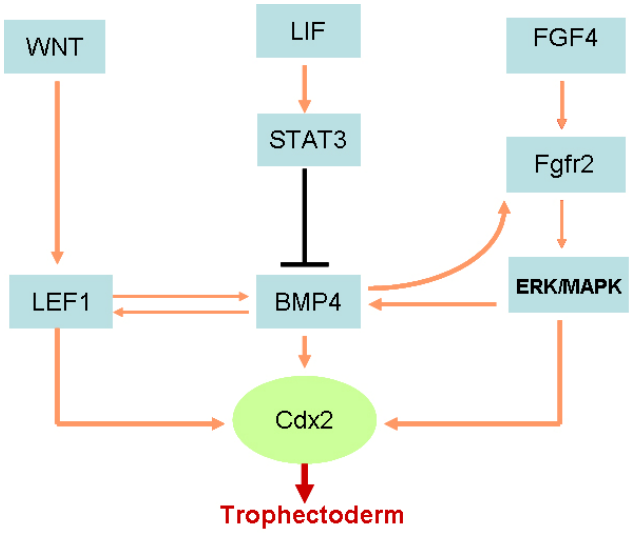


Fig 9 A possible crosstalk between BMP and other pathways involving in trophectoderm specification. Orange arrows indicate activation or induction, while black line indicates inhibition.

In summary, at least three different signaling pathways are likely to be involved in trophoblast differentiation, BMP4/LEF1, WNT/LEF1 and FGF/ERK (Figure 9). Future work will need to establish how they work together to regulate this process.

5.3.4 Spontaneous differentiation of primitive endoderm and trophoblast

Differentiation of primitive endoderm is trigged by aggregation in the outer layer of mESCs regardless of the presence of LIF (Murray and Edgar, 2001; Shen and Leder, 1992). It was then not a surprise that a small proportion of cells in BMP4 cultures express primitive endoderm markers HNF4α.

Grb2 links phosphotyrosine to intracellular signaling pathways, such as FGF (Kouhara et al., 1997). Grb2 is involved in the formation of the primitive endoderm (Cheng et al., 1998). Targeted disruption of Grb2 inhibited primitive endoderm formation in blastocysts (Kouhara et al., 1997). Furthermore, Grb2 links to the activated RAS-ERK

pathway (Kouhara et al., 1997) to regulate primitive endoderm differentiation via repressing Nanog expression (Hamazaki et al., 2006). However, high level Ras signalling promotes trophoblast fate specification while low level Ras promotes both trophectoderm and primitive endoderm differentiation (Lu et al., 2008). Thus Ras may participate in mESCs differentiation via a dosage-dependent manner, and be regulated by different upstream pathways in multiple lineages specification.

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Ying's work showed that the LIF-STAT3 pathway and BMP-Smad-Id pathway collaborate to maintain pluripotency (Ying et al., 2003a). When BMP and LIF are removed most mESCs will develop into neuroectoderm. In this work it was interesting that a percentage of cells cultured in N2B27 media expressed primitive endoderm markers or trophectoderm markers. This shows that although the majority of cells follow the neural lineage, as previously described, a minority differentiate towards primitive endoderm and trophoblast cell fates.

5.3.5 Trophoblast formation in mouse and human ESCs

Although human and mouse ES cells are both blastocyst-derived, they differ significantly in several aspects (Xiao et al., 2006; Ogawa et al., 2007), for example, in the ways they maintain pluripotency (Xu et al., 2002; Pera et al., 2004).

mESCs require LIF and BMP4 to maintain self-renewal, but not feeder layers (Niwa et al., 1998). hESCs self-renewal requires mouse embryonic feeder cells, or a conditioned medium from feeder cells (reviewed by Sato et al., 2004). However, even with serum, LIF and Stat3 are not sufficient to support hESCs self-renewal (Humphrey et al., 2004; Sumi et al., 2004). Instead, FGF collaborates with

activin/nodal signaling to maintain the pluripotency of hESCs in serum-free conditions (Vallier et al., 2005).

Compared with mESCs, hESCs are more like primed mouse epiblast stem cells (mEpiSCs), which are derived from the late epiblast layer of the post-implantation mouse embryo (Tomas JA, 1998). They share flattened cell morphology, and their self-renewal relies on TGF-β/Activin rather than LIF/Stat3 (Xu et al., 2008). Moreover, EpiSCs and human ES cells have closer gene expression profiles than to mESCs (Loh et al., 2006; Brons et al., 2007).

BMP4 is able to promote hESCs to differentiate into trophoblast in a dose-dependent manner (Xu et al., 2002; Schulz et al., 2004). mEpiSCs are also able to differentiate into trophectoderm when treated with BMP4 (Kee et al., 2009). In contrast mESCs were thought to be unable to produce trophoblast, and the ability to form trophoblast in response to BMP4 has been considered one of the differences between human and mESCs (reviewed by Rossant, 2008). This project demonstrated that BMP4 is sufficient to promote trophectoderm differentiation, correcting this misconception.

The close relationship between hESCs and mEpiSCs indicates that hESCs maybe equivalent to the early postimplantation epiblast (Rossant J, 2008). However, mESCs are thought to be comparable to the ICM of early blastocysts. The ICM is closer than the postimplantation epiblast to the developmental stage at which trophoblast cells separate from other lineages. For this reason it is perhaps not surprising that mESCs are able to form trophoblast cells if hESCs and mEpiSCs are able to.

5.3.6 BMP4 in mouse development

This report demonstrated a role for BMP signaling in trophectoderm formation in mESCs. Thus, there is a conserved function of BMP4 in promoting the trophoblast lineage in mESCs, mEpiSCs and human ES cells (Schulz et al., 2004; Xu et al., 2002). A key question is whether BMP signaling is also promoting trophectoderm formation *in vivo*. Is mESC a useful model for a mouse embryo? A role for BMP signaling *in vivo* is supported by the fact that BMP4 is expressed in the polar trophectoderm, while knockouts indicate a role for BMP4 in the extraembryonic ectoderm which is a trophoblast lineage (Coucouvanis and Martin, 1999). A role for BMP signaling in trophectoderm formation *in vivo* could be investigated in a number of ways, an initial experiment could look at the expression of the direct BMP target Msx1 and see if it is expressed in the trophectoderm.

BMP signaling can promote primitive endoderm formation in embryoid bodies (Coucouvanis and Martin, 1999) and monkey ESCs (Kobayashi et al., 2008), consistent with this observation Smad4-knockout embryos are defective in primitive endoderm differentiation (Sirard et al., 1998). Recently it has been shown that the trophectoderm secreted BMP may regulate growth of the ICM (Murohashi et al., 2010). Rather than BMP signaling promoting a single lineage, it appears BMP signaling may regulate multiple lineages. Addressing how signals from the BMP receptors integrate to promote the development of multiple embryonic lineages will be an interesting area for future analysis.

6 Chapter VI Final Discussion and Future work

6.1 BMP4 in the PSE of different vertebrates

The fates of the superficial and deep cells differ among vertebrates, but the early development of different vertebrates shares some similarities, for example, in the mechanisms which generate the two layers (Chalmers et al., 2003; Johnson and Zoimek, 1981; Kimmel and Law, 1985) and the mechanisms which control polarity in the superficial layer (Chalmers et al., 2005; Eckert et al., 2004; Muller, 2001; Plusa et al., 2005). This report demonstrated another shared similarity, that BMP signalling plays a conserved role in promoting differentiation of the first epithelium in *Xenopus* and mESCs by activating Msx/Grhl.

It is tempting to speculate that this pathway will function in the PSE of all vertebrates, but this maybe an oversimplification. *Xenopus* superficial cells express epidermal cytokeratin genes and their expression is regulated by BMP4, but epidermal cytokeratin in the zebrafish EVL does not respond to BMP4 (Sagerström et al., 2005). This suggests there are species differences between the zebrafish EVL and the *Xenopus* superficial layer.

A possible explanation for the similarites between the *Xenopus* superficial layer and the mouse trophoblast is that they are evolutionarily homologous, the complex trophoblast could have evolved from a common ancestor with a simple external epithelium like the *Xenopus* superficial layer. However there is an alternative explanation. BMP signaling is involved in the differentiation of adult epithelia for example in the colon (Deng et al., 2007; Kim et al., 2002; van de Wetering). Msx and

Grhl are also involved in the development of adult epithelia (Ting et al., 2005; Yu et al., 2006). It might be that a similar BMP-Msx-Grhl pathway is used in multiple different types of epithelial cells and that this is why there are similarities between early epithelial cells in *Xenopus* and mouse. This would argue that the superficial cells in *Xenopus* and mouse are not homologous structures.

It appears that BMP4 may not function in promoting the PSE in Zebrafish (see above). Future experiments could examine this issue and also see if there is any role for Msx or Grhl in Zebrafish PSE development. This would establish if similar pathways function in the three vertebrates.

Finally, future experiments could examine if a similar pathway functions in adult epithelia, like the colon. This would establish if the function of this pathway is restricted to the development of embryonic PSE or it has the potential to be reused in many different epithelial types. This might help in our understanding of the development of adult epithelia. It might also allow a comparison of the development of the superficial layer in *Xenopus*, the trophoblast in mouse and different adult epithelia. If the development of the PSE in *Xenopus* and mouse was more similar to each other than to other adult epithelia it might argue that the PSE in different vertebrates is a homologous structure.

6.2 BMP and epithelial cancer

As mentioned in the introduction, BMP4 is involved in epithelial tumorigenesis.

Aberrant regulation of Msx has also been linked to tumour formation (Satokata and Maas 1994; Park et al., 2005). For example, Msx2 exerts a repressive effect on tumour cell growth (Hamada et al. 2005) and Msx1 induces apoptosis in cancer cells (Park et al., 2005). This kind of effect may be due to Msx's role in epithelial differentiation. To understand the role of these genes in tumourigenesis it is important to understand how they function normally to promote the differentiation of epithelial tissues. Thus, understanding the function of Msx in epithelial specification, for example, to understand how Msx regulates tight junction gene expression, becomes necessary.

6.3 Cell polarity and cell migration

In addition to sharing a conserved signaling pathway, a second common point between the *Xenopus* and mouse data is the lack of cell polarity in the BMP4 treated deep layers of *Xenopus* embryos and the inside cells of mESC colonies cultured in BMP4.

By investigating E- and N- cadherin expression in mESCs, Spencer and co-workers demonstrated that spontaneous differentiation of mESCs involves an E- to N-cadherin switch (Spencer et al., 2007). The loss of E-cadherin in some mESCs is linked to EMT (epithelial-to-mesenchymal) (Behr et al., 2005; Spencer et al., 2007) which is amenable to cell movement. In mESCs, BMP4 promoted differentiation triggers the loss of E-Cadherin at the beginning but cells regain expression after differentiation (this report). This suggests that differentiating mESCs would exhibit increased motility, consistent with the observation of the epithelial cells spreading in the report.

In *Xenopus*, E-cadherin is found in superficial and deep layers of the non-neural ectoderm (Levi et al., 1991). Thus, E-cadherin has not been investigated in the report as it is not a good superficial marker. By investigating the localization of ZO1 it was shown that *Xenopus* deep cells do not establish the tight junctions. To some extent, *Xenopus* deep cells are similar to the inner mESCs, remaining at an undifferentiated stage. It raises the possibility that the lack of polarity in *Xenopus* deep cells may reflect increased motility. Experiments testing cell motility of *Xenopus* deep cells and inner cells from mESCs could be carried out.

6.4 Future work and unanswered questions

This report demonstrated that BMP4 promotes a conserved pathway in the first epithelium in *Xenopus* embryos and mESCs, although there remain some details which need to be clarified.

In *Xenopus*, Grhl3 is sufficient to promote superficial differentiation (Chalmers 2006). Msx is a direct target for BMP4 and Grhl1 is an indirect target (Tao et al., 2005). However, it has not been shown that *Msx* can activate *Grhl* expression. It has also not been shown what stops BMP4/Msx promoting differentiation in the deep cells. These issues were considered in detail in Chapter 4.

BMP4 promotes expression of a broad range of trophoblast lineage genes in mESCs. However it is not clear if/how Msx and Grhl are involved in trophectoderm differentiation. The key trophectoderm gene *Cdx2* may be promoted by Msx/ Grhl. Culturing Msx knock-down mESCs in N2B27 media with BMP4, and investigating

the expression of Cdx2 will clarify the relationship between Msx and Cdx2. This was considered in Chapter 5. Since BMPs initiate trophoblast differentiation in hESCs, it would also be interesting to study the role of Msx/Grhl in hESCs.

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